REGULATION OF THYROTROPIN MRNA EXPRESSION IN RED DRUM, *SCIAENOPS OCELLATUS*

A Dissertation

by

RICHARD ALAN JONES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2012

Major Subject: Zoology

Regulation of Thyrotropin mRNA Expression in Red Drum, *Sciaenops ocellatus*

REGULATION OF THYROTROPIN MRNA EXPRESSION IN RED DRUM, *SCIAENOPS OCELLATUS*

A Dissertation

by

RICHARD ALAN JONES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Duncan S. MacKenzie
Committee Members,	John T. Jaques
	Delbert M. Gatlin III
	Brian D. Perkins
Head of Department,	U.J. McMahan

August 2012

Major Subject: Zoology

ABSTRACT

Regulation of Thyrotropin mRNA Expression in Red Drum,

Sciaenops ocellatus. (August 2012)

Richard Alan Jones, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Duncan S. MacKenzie

The role of thyroid-stimulating hormone (TSH) in the regulation of peripheral thyroid function in non-mammalian species is still poorly understood. Thyroxine (T_4), the principal hormone released from the thyroid gland in response to TSH stimulation, circulates with a robust daily rhythm in the sciaenid fish, red drum. Previous research has suggested that the red drum T_4 cycle is circadian in nature, driven by TSH secretion in the early photophase and inhibited by T_4 feedback in the early scotophase. To determine whether TSH is produced in a pattern consistent with driving this T_4 cycle, I developed quantitative real time RT-PCR (qPCR) techniques to quantify the daily cycle of expression of the pituitary TSH subunits *GSU*α, and *TSH*β. I found that pituitary TSH expression cycled inversely to, and 6-12 hours out of phase with, the T_4 cycle, consistent with the hypothesis that TSH secretion drives the T_4 cycle. To examine the potential role of deiodinases in negative feedback regulation of this TSH cycle, I also utilized qPCR to assess the pituitary expression patterns of the TH activating enzyme outer-ring deiodinase (Dio2) and the TH deactivating enzyme inner ring deiodinase (Dio3). Whereas *Dio2* was not expressed with an

obvious daily cycle, *Dio3* was expressed in the pituitary mirroring the TSH cycle. These results are consistent with T_4 negative feedback on TSH and suggest that TH inactivation by pituitary cells is an important component of the negative feedback system. To further examine the TH regulation of this *Dio3* cycle, I developed an immersion technique to administer physiological doses of T_3 and T_4 *in vivo*. Both hormones persist in static tank water for at least 40 hours. Immersion in 200ng/ml T_4 significantly increased both plasma T_4 and T_3 within physiological ranges above control at 4.5 hours. Immersion in 100ng/ml T_3 increased plasma T_3 within physiological ranges over control by 22 hours while significantly decreasing plasma T_4 below control, presumably through inhibition of TSH secretion. T_4 also significantly inhibited the expression of the TSH α and β subunits at 4.5 and 22 hours of immersion whereas T_3 immersion significantly inhibited the expression of the α and β subunits of TSH by 22 hours. Both *Dio2* and *Dio3* expression were significantly diminished by T_3 and T_4 at 22 hours. Inhibition of circulating THs with the goitrogen methimazole significantly increased the expression of TSH. These results indicate that both T_4 and T_3 are capable of negative feedback regulation of TSH expression in red drum on a time scale consistent with the T_4 daily cycle, and further support Dio3 destruction of THs in the pituitary, potentially regulated by circulating T_4, as a critical component of negative feedback on TSH. This study supports the importance of central mechanisms acting through pituitary TSH secretion in regulating thyroid function in red drum.

DEDICATION

For my family and friends

ACKNOWLEDGEMENTS

I thank my major professor, Dr. Duncan MacKenzie, for his mentorship, his intellectual advice and experimental assistance on my project, and for his professional friendship and understanding. I would like to thank Dr. Delbert Gatlin III and Dr. Scott Jaques for their scholarly contributions and assistance with animal sampling. I further thank Dr. Scott Jaques of the Texas Veterinary Medical Diagnostic Laboratory for providing facilities and reagents for performing RIAs. I also thank my final committee member Dr. Brian Perkins for his insightful contributions that led to productive changes in my project. Additionally, I thank Dr. William Cohn for assistance in animal sampling, intellectual and physical contributions to experiments including techniques, and his professional friendship. I greatly appreciate the help Dr. Kevin Leiner gave me when teaching me experimental design and in the development of the qPCR assays used throughout my studies. I thank Dr. William Cohn, Dr. Kevin Leiner, and Dr. Roldan Valverde for their work in cDNA cloning the full length red drum *TSH*β. I thank Dr. Thomas Miller, Brian Ray, Allison Wilkes, and Rhonda Patterson of Texas A&M University and the Dow Chemical Company for assistance in obtaining and maintaining fish. I also thank Dr. Ketan Patel and Dr. Phil Brand for their assistance with molecular biology techniques. I also thank the Lekven, Riley, McKnight, and Thomas labs in the Biology Department at Texas A&M University for advice and use of facilities. I thank Dr. Jack McMahan for

mentoring me in the skill of verbal research presentation. I wish to acknowledge the contributions of Alison Cook, John McElroy, Cristina Copus, Elizabeth Drone, Quyhn Lam, Julie Butler, Jarrett Aldinger, Bryan Berletch, and Laura Ron as undergraduate researchers working on this project. I thank the Department of Biology at Texas A&M University for employment while I worked on this project. I also acknowledge my wife Jennifer Jones. Funding for this project was provided in part by the Department of Biology and the Graduate Interdisciplinary Program in Marine Biology, both at Texas A&M University.

TABLE OF CONTENTS

Page

LIST OF FIGURES

FIGURE Page

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Background

All vertebrates possess a thyroid gland which produces iodinated thyroid hormones, principally the prohormone 3,5,3',5'-tetraiodothyronine (thyroxine, T_4) [21]. T_4 is transported into target cells by organic anion transporter proteins where it can be deiodinated by the type 2 deiodinase enzyme (Dio2) to the active hormone 3,5,3'-triiodothyronine (T_3) or type 3 deiodinase enzyme (Dio3) to inactive thyroid hormone metabolites [76]. T_3 binds to nuclear thyroid hormone receptors to regulate gene transcription [64]. Thyroid hormones (THs) then act directly or through permissive interactions at target cells to regulate numerous important physiological processes including reproduction, development, growth, and metabolism [17, 34, 45, 51, 75, 93].

In mammals, T_4 secretion is principally controlled by thyrotropin (TSH), a heterodimeric glycoprotein hormone produced by the pituitary gland [52]. TSH comprises two subunits: an α subunit (glycoprotein hormone subunit α, GSUα, shared with the gonadotropins) that is necessary for holoprotein stability

---

This dissertation follows the style of General and Comparative Endocrinology.

and activity and a β subunit that conveys the thyrotropin-specific action of the hormone [83]. The heterodimer binds to TSH receptors (TSHR) in the thyroid gland stimulating increased cAMP levels which activate thyroid hormone synthesis and release [64]. Mammalian TSH is in turn regulated primarily by thyroid hormone negative feedback and hypothalamic thyrotropin-releasing hormone (TRH) [11]. Because of the primary importance of the hypothalamus and pituitary in determining thyroid hormone availability to target cells, Eales and Brown [21] characterized this mammalian model as the central model of regulation of thyroid function (figure 1.1).

Whereas mammalian TSH has been well studied, less is known about TSH from nonmammalian species. Sea lampreys, representing the oldest extant vertebrate lineage, appear not to possess TSH in the form seen in higher vertebrates, but instead have one or two glycoprotein hormones that may control both thyroid function and reproduction [82]. No consistent evidence proves the presence of a TSH in elasmobranchs. On the other hand, the amino acid sequence has now been described for over a dozen teleost fish TSHβs [55]. Regions of mammalian TSH found to be important for receptor activation and α-β subunit interaction are well conserved in known fish TSHβ and GSUα sequences suggesting these regions are functionally similar in fish and mammals [33, 55, 60, 78, 83].

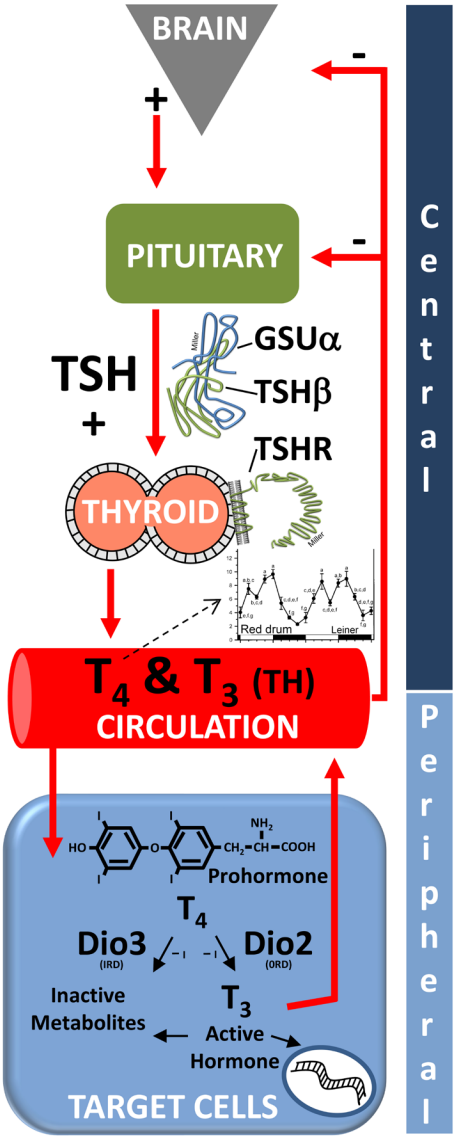

Figure 1.1. Central versus peripheral regulation of thyroid function. The structural representations of TSHβ, GSUα, and TSHR displayed in this figure were originally drawn by Miller [60]. The T_4 daily cycle displayed in this figure was originally graphed by and typical of data recorded by Leiner [46]. I generated all other aspects of this figure for use in this dissertation. The central regulation of thyroid function begins at the brain where signals, still unknown in fish, stimulate and/or inhibit TSH release from the pituitary. TSH is composed of a thyroid specific β subunit and a shared α subunit. TSH travels through the circulation and binds to membrane receptors on thyroid follicles dispersed throughout the lower jaw of most fish to stimulate the thyroid to release the prohormone T_4. Evidence suggests that THs inhibit the expression of TSH at the pituitary. In mammals, circulating T_4 drives TH provision to peripheral tissues. Thyroid activation of targets can also be regulated peripherally when T_4 is taken into target cells and converted to either the active hormone T_3 by the outer-ring deiodinating enzyme Dio2 or inactive TH metabolites by the inner-ring deiodinating enzyme Dio3.

While the sequences of fish TSHβ and GSUα have been well characterized, the functional significance of fish TSH is still poorly understood. It has been noted that, while TSHβ immunoreactive cells are present in fish pituitaries, they comprise a relatively small proportion of the gland [39, 92]. Furthermore, negative feedback of THs on thyroid function, an important component of central thyroid regulation [21], has not been consistently observed at physiological concentrations and time course [21, 55]. Given that TSH originated near or before the evolutionary divergence of gnathostomes from other vertebrate groups and that thyroid hormones most likely originated as exogenously secreted or ingested regulatory signals, some researchers, most notably Eales and Brown [21], have concluded that thyroid function in fish reflects a more primitive peripheral control in which thyroid hormone supply to targets is primarily regulated through deiodination in peripheral tissues rather than centrally-driven from the hypothalamus [19, 21]. In this peripheral model of regulation of thyroid function, deiodinase control of T_3 availability to target cells is rate limiting as opposed to the central model in which TSH drives T_4 availability and utilization in target cells (figure 1.1).

Recent evidence suggests that central regulation of thyroid function may be more important in fish than originally proposed by Eales and Brown [21]. Recent studies have shown that fish TSH production is responsive to stimulation by hypothalamic hormones, suppressed by TH negative feedback, and elevated during major life history events, as reviewed by MacKenzie et al. [55]. Together

these observations suggest that TSH may be dynamically regulated by central mechanisms. However, most TSH studies in fish have focused on structure, primarily examining TSH mRNA expression in cold-water salmonid species with relatively low circulating TH levels [55]. Furthermore, relatively little information is available on the physiological regulation of TSH production in any teleost species.

In particular, fish TSH studies have failed to address several critical questions raised by Eales and Brown [21] that would provide support for a central model of thyroid regulation in fish, including the importance of T_3 from the periphery versus T_4 from thyroid gland origin in regulating negative feedback at the pituitary, the role of deiodinase at the pituitary in regulating thyroid hormone feedback, or the importance of TSH production and TSH receptor binding in driving thyroid secretion of T_4, which can subsequently be converted to T_3 in target cells. Establishing that TSH is secreted by the pituitary, regulated by T_4 feedback, and that TSH binds to TSH receptors to drive T_4 production and release into the blood at times of increased metabolic activity is an essential test of the central model of regulation of thyroid function (Eales and Brown [21]).

In the present study, I addressed the question of the importance of TSH in regulating peripheral thyroid function in fish by examining TSH mRNA expression in a warmwater fish species, the red drum, an important sport and aquaculture species on the Texas Gulf coast. The red drum has dynamic daily

cycles of T_4 in the blood [47] making it an excellent species in which to study T_4 production and utilization. My approach evaluated the relationship between TSH production, deiodinase and TSH receptor expression, and circulating TH levels to test the hypothesis that in the red drum, the production and target cell utilization of T_4 is driven by TSH similar to the manner seen in thyroid function centrally regulated higher vertebrates.

Adult red drum can be collected from the wild and juvenile animals that adapt well to captivity can be obtained from the Texas Parks and Wildlife Stock Enhancement Program. Research with red drum over the last 20 years supports the hypothesis that TSH in this species is dynamically controlled by central mechanisms. Leiner et al. [47] described a robust daily rhythm of circulating T_4 in red drum. This rhythm persisted in constant dim illumination and did not entrain to feeding time though the amplitude of the cycle was decreased in feed restricted animals and by T_3 immersion [48, 49]. Leiner and MacKenzie [49] proposed that the free-running circadian rhythm of circulating T_4 in red drum was controlled by a central mechanism through which food and light activated the hypothalamic-pituitary-thyroid (HPT) axis to produce TSH during the day, driving increasing circulating T_4 levels starting in the morning. Negative feedback then inhibited TSH production during the afternoon. The proposed dynamic role of TSH in regulating these daily cycles of T_4 in red drum made this species an appealing choice for examining the central regulation of thyroid function in teleost fish.

Presently, no reliable immunoassay exists to measure circulating TSH in fish. TSH stimulates T_4 release in goldfish and coho salmon [60, 61], and T_3 inhibits whereas methimazole stimulates *TSH*β mRNA expression and blood TSH, respectively in coho salmon [43, 61], indicating that TSH mRNA expression can serve as an index for TSH production. Conclusions based on measurements of steady-state mRNA expression assume that mRNA expression equates to subsequent translated and secreted protein, an obvious limitation of this approach, but given that TSH protein presently cannot be accurately measured in fish blood, the measurement of mRNA expression in the pituitary provides the best current viable index of thyrotropin activity in fish. Expression of *TSH*β and *GSU*α mRNA has been readily assayed by Northern blots, reverse-transcriptase PCR methods, and real-time qPCR [55]. These techniques were readily adaptable to red drum TSH. Blotting techniques require a relatively large amount of RNA and thus often require pooling several pituitaries to create a single replicate [58, 94]. PCR assays on the other hand require much less RNA, allowing pituitaries to be assayed individually, and increasing the statistical power of experiments while decreasing animal numbers. Real-time qPCR assays are now becoming the standard for pituitary hormone expression studies in fish [13, 30]. For these reasons I developed a real-time qPCR assay for the measurement of TSH subunit expression in red drum. I also developed real-time qPCR assays for the measurement of *Dio2*, *Dio3*, and *TSHR* expression in red drum.

As a first step in examining the importance of centrally regulated TSH in red drum I evaluated basal expression of mRNAs for *TSHβ, GSUα, Dio2, Dio3,* and *TSHR* in the pituitary gland and correlated these values to those of circulating TH levels on a daily and seasonal basis. If TSH is a central driver of thyroid function in red drum, I expected TSH expression to precede, with a similar pattern, circulating T_4 levels. Most studies of TSH expression in fish have focused on hypothalamic control or negative feedback and rarely address the relationship between TSH expression and circulating TH levels as reviewed by MacKenzie et al. [55]. A few studies have found that changes in TSH mRNA expression correspond with life history events. For example, it has been found that TSH expression and circulating T_4 are elevated leading up to or during metamorphosis in the Japanese eel and Sengalese sole, parr-smolt transformation in Atlantic salmon, and spawning in goldfish [30, 56, 58, 81], suggesting that the thyroid may play an important role in these processes. However, no study has examined TSH expression associated with dynamic T_4 cycles such as those observed in red drum. Data from these initial experiments tested the hypothesis that the daily T_4 cycles in red drum are driven centrally by the pituitary, while simultaneously provided baseline expression data used for subsequent studies of TSH, deiodinase, and *TSHR* expression.

To further examine the importance of central regulation of thyroid function in red drum I next evaluated the negative feedback of TSH production. Negative feedback of T_4 and T_3 at the pituitary gland is a key component of the central

model for regulation of thyroid function [21]. Since thyroid hormone receptors primarily bind T_3 and not T_4 [64], and deiodinase activity can either activate T_4 to T_3 or inactivate T_4 and/or T_3 [21], the expression of deiodinases in the pituitary is required if central mechanisms acting through T_4 drive thyroid hormone cycles of utilization in peripheral cells and in feedback regulation of TSH production at the pituitary itself. If T_3 produced and released from peripheral cells, and not T_4 from the thyroid gland, drives fish thyroid function, then target cells, including pituitary thyrotrophs, should be relatively unresponsive in TSH and deiodinase expression to T_4 but readily regulated by T_3. Indeed liver deiodinase expression in trout is regulated by T_3 and not T_4 [6]. Consistent with mammalian studies, T_4 or T_3 negative feedback control of TSH has been observed in nonmammalian studies [12, 28, 55, 57] but has been rarely studied in fish, leaving the dynamics and mechanisms of said regulation, presumably through deiodinases, unknown. Studies have shown that Dio2 cycles daily in the rat pituitary and that Dio2 mediates TH negative feedback [14, 54, 62]. *Dio3* is expressed and positively regulated by T_3 in the mouse pituitary [2], but no studies have addressed the intra-pituitary regulation of Dio2 and Dio3, or the cyclicity of Dio2 in the fish pituitary, nor pituitary Dio3 cyclicity in any species. A pituitary T_3 negative feedback mechanism that chronically suppresses TSH expression in a dose dependent manner at low concentrations *in vitro* has been observed in the European eel [74]. A similar pattern of T_4 negative feedback has been observed *in vitro* in the bighead carp [13]. These studies provide evidence that negative

feedback is important for the regulation of fish thyroid function. However, the issue of rapid central regulation through both T_4 and T_3 *in vivo* has not been addressed. Red drum provided an excellent opportunity to address this question because T_3 negative feedback on circulating T_4 had already been described *in vivo* in this species [49]. Using the knowledge of how TSH expression changes throughout the day in red drum I chose optimal times to test suppression of TSH expression and regulation of deiodination in the pituitary in a negative feedback study.

The TSHR is expressed, in many species, in locations other than the thyroid gland [4, 55]. This non-thyroidal expression is hypothesized to play a more primitive paracrine signaling role separate from the role of the receptor for activating thyroid hormone production in the thyroid gland [4]. In the European hamster, *TSHR* is expressed in the pituitary and cycles with photoperiod consistent with a role in seasonal reproduction [32]. The *TSHR* is also expressed in the chicken pituitary and functions in a local negative feedback loop [29] of unknown significance in other species. *TSHR* expression has been localized to the African catfish pituitary but the functional significance of the receptor was not addressed [87]. Seasonal cycles of the TSHR in the fish pituitary would suggest a central nervous system-TSH role in seasonally-regulated activities such as growth or reproduction. A paracrine pituitary TSH feedback through the TSHR in which the TSHR cycles independently of circulating T_3 or T_4 would represent a highly centralized regulation of thyroid

function where the central nervous system drives TSH into the blood without input from the periphery.

Objectives

This research tested the hypothesis that in the red drum, the production of TSH drives the subsequent production of T_4 from the thyroid gland to regulate thyroid function in a manner similar to the central regulation of thyroid function seen in higher vertebrates. To test this hypothesis my objectives were:

1) To clone and characterize the sequences of red drum *TSHβ, GSUα, Dio2, Dio3*, and *TSHR* mRNA.
2) To develop and biologically validate assays to measure red drum *TSHβ, GSUα, Dio2, Dio3*, and *TSHR* mRNA expression.
3) To characterize the relationship between basal *TSHβ, GSUα, Dio2, Dio3*, and *TSHR* mRNA expression patterns and circulating TH levels in red drum.
4) To characterize the negative feedback regulation of red drum *TSHβ, GSUα, Dio2, Dio3*, and *TSHR* mRNA expression.

By evaluating the temporal pattern and negative feedback regulation of TSH in red drum, a teleost fish known to have dynamic cycles of circulating T_4, these studies helped elucidate the contribution of central mechanisms to the regulation of thyroid function in teleost fish.

CHAPTER II

EXPRESSION OF *TSHβ, GSUα, DIO2, DIO3,* AND *TSHR* mRNA IN CAPTIVE AND WILD RED DRUM, *SCIAENOPS OCELLATUS*

Introduction

The relative importance of central versus peripheral regulation of thyroid function in fish has been debated [21, 55]. Thyroid function is regulated both by signals originating from the central nervous system acting through TSH and by metabolic pathways for hormone activation and inactivation acting through deiodinase activity in peripheral target cells [21]. The primary stimulatory importance of hypothalamic thyrotropin-releasing hormone (TRH) and pituitary TSH in determining active thyroid hormone availability to target cells in mammals led Eales and Brown [21] to characterize this mammalian model as the central model of regulation of thyroid function. The central pathway results in release of TSH (a heterodimer of the glycoprotein subunits GSUα and TSHβ) from the pituitary. TSH then binds to the TSH receptor (TSHR) on the thyroid gland to drive T_4 production and subsequent triiodothyronine (T_3) availability to target cells in mammals [21]. The cascade continues as thyroid hormone receptors primarily bind T_3 and not T_4 to elicit the target cell response [64]. Given that TSH originated near or before the evolutionary divergence of gnathostomes from other vertebrate groups [82] and that thyroid hormones most

likely originated as exogenously secreted or ingested regulatory signals [19], some researchers, most notably Eales and Brown [21], have concluded that thyroid function in fish reflects a more primitive peripheral control in which thyroid hormone supply to targets is primarily regulated through deiodination in peripheral tissues rather than centrally-driven from the hypothalamus and pituitary [19, 21]. In this peripheral model of regulation of thyroid function, deiodinase control of T_3 availability to target cells is rate limiting as opposed to the central model in which TSH drives T_4 availability and utilization in target cells.

Peripheral mechanisms for regulating thyroid function work through outer-ring deiodinating (ORD) enzymes such as deiodinase 2 (Dio2) and inner-ring deiodinating (IRD) enzymes such as deiodinase 3 (Dio3) [21]. Dio2 is an activating enzyme when converting T_4 to T_3 while Dio3 is a TH inactivating deiodinase [21]. The relative activity of these deiodinase enzymes then controls T_3 availability in targets [21]. Dio2's ability to generate intracellularly active thyroid hormone makes this enzyme a critical component of any thyroid-responsive system, including thyroid hormone activation of target cells and thyroid hormone negative feedback in the pituitary [14]. Negative feedback from T_4, and intra-pituitary Dio2-produced T_3, are essential components of the central regulation of TSH secretion [21]. The significance of Dio3 in thyroid function is less well understood, but its ability to deactivate thyroid hormones in target cells

appears to be an important mechanism for protecting sensitive tissues such as the central nervous system from excessive thyroid hormone stimulation [6, 65].

Because circulating levels of thyroid hormones in fish can vary dynamically on a daily or seasonal basis [44, 49, 81], it would be expected that if negative feedback were regulating TSH secretion pituitary TSH production and deiodinase activity would also cycle in a fashion consistent with inhibition of TSH release during periods of elevated circulating thyroid hormones. In support of an important role for TSH in driving thyroid hormone production, TSH mRNA transcripts have been shown to fluctuate over time or during critical metamorphic and reproductive stages associated with thyroid activation in several fish species [15, 30, 56, 81]. Additionally, T_3 has been found to inhibit TSH expression, suggesting an active negative feedback system [55]. However, *in vivo* studies of fish TSH expression have generally been undertaken with pharmacological doses or time courses of T_3 only [55], failing to establish that feedback inhibition of TSH occurs rapidly (hours rather than days) through physiological levels of circulating T_4 and T_3, as would be expected in a dynamically-regulated central system [6]. Furthermore in teleosts, there has been no demonstration of changes in expression of pituitary deiodinase enzymes serving to supply thyroid hormone to thyrotrophs in a manner consistent with achieving negative feedback, as would also be expected in a central model [6]. A necessary step in examining the central regulation of fish thyroid function is therefore establishing if the required components of negative feedback systems exist in the fish

pituitary, and if they cycle in a fashion consistent with negative feedback when circulating thyroid hormones are elevated.

The red drum (*Sciaenops ocellatus*) is an important sport fish species on the Texas Gulf coast. Because of its aquacultural potential, the red drum has been the subject of numerous nutritional, ecophysiological, and endocrinological studies [49, 59, 69, 86]. Studies of the hormonal regulation of growth have suggested that the red drum may serve as a useful fish species for a more detailed investigation of the regulation of thyroid function. In captivity, red drum exhibit a dynamic daily cycle of thyroxine (T_4) in the blood [47]. In the rat, circulating TSH precedes the daily T_4 cycle by approximately four hours suggesting that this T_4 cycle is driven by alternating periods of activation of TSH stimulation of the thyroid gland followed by TSH inhibition [10]. Similarly, it has been proposed that the T_4 cycle in red drum is regulated by a reciprocal interaction between central activation of thyrotropin (TSH) secretion in the morning versus feedback inhibition in the evening [49]. This dynamic cycle suggests that the red drum could serve as a useful model species in which to examine the contributions of central control to the maintenance of basal thyroid function in a teleost fish.

If the daily T_4 cycle in red drum is indeed regulated through TSH expression and driven in part by negative feedback, this species provides an excellent opportunity to examine the relative importance of deiodinases in the

regulation of TSH production in a teleost fish species. In some long day breeding mammals and at least one bird, reciprocal cycles of hypothalamic *Dio2* and *Dio3* during long or short photoperiods coupled with increased TSH expression from non-thyrotroph pituitary cells drives T_3 availability during alternating photoperiods to regulate gonadotropin release for seasonal reproduction [31, 32, 90]. Additionally, Dio2 cycles in the rat pituitary are thought to set the circadian rhythm of TSH by regulating the amount of T_3 available for negative feedback [54, 62], further supported in mice, where free T_4 and Dio2 regulated T_3 production negatively correlate [14]. These data suggest Dio2 as a likely candidate for a central regulator of thyroid hormone availability for negative feedback in thyrotrophs. However, recent studies in mammals have also suggested that Dio3, through its catabolic actions, may be equally important in releasing central mechanisms from feedback inhibition [2, 76]. Daily TSH cycles, such as those proposed in the red drum, may thus result from activation of T_4 feedback via Dio2 production of T_3 followed by diminished feedback achieved through activation of Dio3. At present, no information is available on the presence of these deiodinases in the fish pituitary in association with daily thyroid hormone cycles.

The dynamic cycle of circulating T_4 in red drum was hypothesized to be driven centrally by TSH early in the photophase and down regulated by negative feedback late in the photophase, [49] but the mechanism through which this is achieved has not been elucidated. I hypothesize that if the previously described

daily T_4 cycle in red drum is truly due to TSH stimulation of the thyroid in the morning, elevations of circulating T_4 will be preceded by increased expression of pituitary *TSH*β and *GSU*α. I further hypothesize that *Dio2* will be expressed in the pituitary in phase with T_4, thus supplying T_3 for feedback. Lastly, I hypothesize that if *Dio3* is expressed in the red drum pituitary, its cyclicity should be consistent with a role in diminishing T_4 feedback as TSH expression is increasing. The objectives of this study were therefore to characterize the daily rhythms of thyroid related genes in the red drum pituitary and compare these rhythms to that of circulating T_4. By development of novel, real-time quantitative PCR (qPCR) techniques to quantify mRNA expression, I have the first opportunity to evaluate all of these gene products simultaneously in the same tissue without pooling samples, thus substantially reducing the numbers of animals required. Additionally, because of the exquisite sensitivity of qPCR, these studies also have provided an opportunity to examine the expression of the *TSHR* in the red drum pituitary and determine whether its expression pattern suggests a role in regulation of TSH expression. Whereas the TSHR has traditionally been thought to be exclusively expressed on thyroid epithelial cells, recent studies have identified other locations where it may serve a regulatory function. For example, hypothalamic TSHR may be important in reproductive seasonality, where it serves as a link in paracrine pituitary TSH regulation of the hypothalamus [31]. TSHR may also have a paracrine role in reproduction where TSH signaling is suggested to stimulate final gamete maturation and gonadal

development [27, 55, 71, 89], as well as in the gut where local TSH and TSHR regulate the activity of intestinal lymphocytes [88]. Of particular interest, ectopic expression of TSHR in the pituitary has been proposed to play a role in negative feedback on TSH by binding TSH and activating local T_3 production [29]. The present study provided a unique opportunity to determine whether the TSHR may also be involved in negative feedback on TSH in fish. Finally, to more fully characterize ectopic expression of thyroid-related genes, I was also able to obtain tissues from wild red drum which provided larger quantities (liver, intestine, pituitary) of tissue, as well as adult gonads, not available in our laboratory-reared juveniles.

Materials and methods

Animals

The CCA/CPL Marine Development Center in Corpus Christi or the Texas Parks and Wildlife Department's hatchery at Sea Center Texas in Lake Jackson provided red drum fingerlings which were reared in the Department of Biology's BioAquatics Facilities at Texas A&M to at least 20g before use. Animals were housed in a 4000L recirculating system at 4ppt salinity (SuperSalt, Fritz Industries, Mesquite, Texas), 26C, 12L:12D photoperiod. Juvenile fish were fed Aquamax (PMI Nutrition, Brentwood, MO) to apparent satiation once daily. Wild red drum were angled from waters of the Gulf of Mexico adjacent to the Dow Chemical facility in Freeport, Texas as described by Craig et al. [16]. Wild red

drum (20-28 inches) were caught and sampled in the afternoon during sampling trips in April and August of 2007, May of 2008, and July of 2009. Fish were euthanized in MS-222 (Finquel, Argent Laboratories, Redmond, Washington) prior to obtaining blood and tissue samples. Several tissues, including pituitaries, gonads, liver, and intestine, were removed immediately upon capture and frozen in TRIzol (Invitrogen) on dry ice for transport back to College Station. Sex and reproductive state were determined by dissection. Testes from April were in early recrudescence. In all other months testes were in early to mid-recrudescence. Ovaries from August were in mid to late recrudescence. In all other months ovaries were in early to mid-recrudescence. No tissues were pooled from wild animals. Six pituitaries, three ovaries and three testes were collected from each sampling trip. Pituitary mRNA expression from these wild animals was compared to expression from 45 juvenile control lab animals (26.65±1.31g average body weight, no tissues were pooled from these lab animals) held under the conditions described above and sampled between 3-8h after lights on. For 24 (21.72±1.40g) of these 45 fish, RNA was extracted from both the liver and pituitary for a tissue specific comparison of deiodinase expression. All procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee.

Daily cycles in lab red drum

Two cycle experiments were performed based on protocols developed by Leiner et al. [47]. For both cycle experiments, animals were fed at random times during the photophase and left undisturbed for 1 month. For the first cycle study, experimental animals were maintained in 6,400L round tanks. 12 animals (100.59±4.14g) from a single tank were sampled per time point every 4h until all tanks had been sampled once. Following euthanasia in MS-222, the dorsal cranium and brain were removed by dissection to expose the pituitary. Pituitaries were pooled into groups of three per treatment for each extraction replicate yielding four pooled samples for each treatment at each time point. During the second cycle experiment animals were housed in 80L rectangular tanks. Eight animals (55.45±1.70g) were sampled from one tank per time point every 3h until each tank had been sampled once. Pituitaries were not pooled. To combat increased mortality in later cohorts of laboratory-reared red drum [70], the animals in this experiment were fed our standard Aquamax diet sprayed with 20mg potassium iodide/kg food as described by Gensic et al. [26].

Blood and tissue analysis

Blood was collected from the caudal vasculature and plasma was separated and frozen at -80C until analyzed for THs using Coat-A-Count Total T_4 or T_3 kits (Siemens, Los Angeles, CA) following the manufacturer's protocol as described by Cohn et al. [15]. Following terminal anesthesia, tissues for RNA

extraction were rapidly removed by dissection and transferred to either TRIzol (Invitrogen) for wild samples, Qiagen Rneasy Plus Mini kit reagent for lab samples of intestines and pituitary pools, or Zymoresearch ZR RNA Microprep kit reagent for pituitaries sampled individually. All samples were frozen at -80C until RNA extraction according to the manufacturers' protocols. Samples were taken from the estimated mid-region for large structures such as the gonad or intestine. GlycoBlue (Invitrogen) was added during the TRIzol extractions to visualize the pellets.

cDNA cloning and sequence analysis of red drum Dio2, Dio3, and TSHR

Red drum *Dio2*, *Dio3*, and *TSHR* internal fragments were generated by PCR using degenerate primers designed by Isorna et al. [35] and Goto-Kazeto et al. [27]. All other primers used in this study were red drum specific. PCR primers are listed in table 2.1. RACE (rapid amplification of cDNA ends) was performed on liver RNA using a GeneRACER kit (Invitrogen) following the manufacturer's protocol. The 5' end of the red drum *Dio2* was amplified from this RACE reaction. PCR products were ligated into recombinant plasmids and transformed into *E. coli* using the TOPO TA Cloning kit (Invitrogen) following the manufacturer's protocol. At least three independent colonies for each PCR product were prepped using the Concert High Purity Plasmid Miniprep kit (GibcoBRL, Life Technologies) and sequenced. BLAST (National Center for Biotechnology Information) was used to identify transcripts of interest from the

sequenced PCR products. Clustal (EMBL-EBI) was used to align sequences and Mega4 software (www.megasoftware.net), using the neighbor-joining method with 1000 replicates, was used to generate phylogenetic trees.

Table 2.1. Oligonucleotide primers. Primer direction and application is indicated. Sequence information from cloned fragments was used to design qPCR primers and probes.

Oligonucleotide Primers

Primer	Nucleotide Sequences	Direction	PCR
TSHβ$_1$	5'-ACTCAAGGGACAGCAACA-3'	Forward	qPCR
TSHβ$_2$	5'-TGAAGACAGGGTTGGAGT-3'	Reverse	qPCR
TSHβ$_3$	5'-56-FAM/TATGACAAG/ZEN/GTGGAATACCGCACAGC/3IABkFQ-3'	Probe	qPCR
GSUα$_1$	5'-CAAACATGGGCTGTGAAGAGTG-3'	Forward	qPCR
GSUα$_2$	5'-GTTCTTTGGGATCGTCATCGTC-3'	Reverse	qPCR
GSUα$_3$	5'-56-FAM/TCCAACACC/ZEN/TCTCAAGGCCATGAA/3IABkFQ-3'	Probe	qPCR
Dio2$_1$	5'-TSCGSTBCRTHTGGAACAGCTT-3'	Forward	Internal Fragment
Dio2$_2$	5'-GGKTGWGCCTCRTCRATGTAGACCA-3'	Reverse	Internal Fragment
Dio2$_3$	5'-CAGAGGGCGATCCGATGACTCAAAAT-3'	Reverse	5' RACE
Dio2$_4$	5'-CGCTCCATCTGGAACAGTTT-3'	Forward	qPCR
Dio2$_5$	5'-CACCTTCACCAGTTTGGAGTTG-3'	Reverse	qPCR
Dio2$_6$	5'-56-FAM/CCTTCTGGA/ZEN/CGCCTACAAACAGGTAA/3IABkFQ-3'	Probe	qPCR
Dio3$_1$	5'-AYGRNCARAARYTGGACTTYYTCAA-3'	Forward	Internal Fragment
Dio3$_2$	5'-TAVGGSGCRTCYGWGCTSACCCAGC-3'	Reverse	Internal Fragment
Dio3$_3$	5'-GTACGGAGCCTACTTTGAGAG-3'	Forward	qPCR
Dio3$_4$	5'-ATCGTTCCTGTATTGCTCCAG-3'	Reverse	qPCR
Dio3$_5$	5'-56-FAM/TCGTGAGGG/ZEN/ATGAGAGAGTGGTGT/3IABkFQ-3'	Probe	qPCR
TSHR$_1$	5'-TTCAAYCCHTGCGAGGAYATHATGGG-3'	Forward	Internal Fragment
TSHR$_2$	5'-GTYTGCCAGTCGATDGCGTGGTTGTA-3'	Reverse	Internal Fragment
TSHR$_3$	5'-CTGGCTATTCTGGGAAATGTGG-3'	Forward	qPCR
TSHR$_4$	5'-CAGAGGCGATAAGCAGCAGATA-3'	Reverse	qPCR
TSHR$_5$	5'-56-FAM/TTCCTCATG/ZEN/TGTCACCTGGCCTTT/3IABkFQ-3'	Probe	qPCR
18S Ribosomal$_1$	5'-CGAAAGTTGATAGGGCAGAC-3'	Forward	qPCR
18S Ribosomal$_2$	5'-AGATCCAAAACCCATGCG-3'	Reverse	qPCR
18S Ribosomal$_3$	5'-56-FAM/AGGTTATCT/ZEN/AGAGTCACCAAAGCGGC/3IABkFQ-3'	Probe	qPCR

PCR

Standard PCR was performed using GoTaq Green Master Mix (Promega, Madison, WI) according to the manufacturer's protocol and guidelines. Standard PCR reactions were passed through gel electrophoresis and visualized under UV light [15]. The samples studied for tissue specific expression were previously described and assayed for *TSH*β, *GSU*α, and actin expression, and shown not to contain genomic DNA contamination, by Cohn et al. [15]. PCR products were sequenced to confirm identity using BigDye reagent (Applied Biosystems/Invitrogen) according to the manufacturer's protocol followed by electrophoresis at the Gene Technology Laboratory at Texas A&M University. All RNA samples were treated with DNAse I (Zymo Research) according to the manufacturer's protocol prior to PCR. qPCR step 1 RT reactions and step 2 PCR reactions were performed using the high-capacity cDNA Reverse Transcription Kit and TaqMan Universal PCR Master Mix (Invitrogen) with red drum specific qPCR primers/probes (Integrated DNA Technologies) following the manufacturer's protocol. qPCR primers/probes were designed using Primer Quest software from Integrated DNA Technologies and designed to cross a predicted intron-exon boundary when possible [9]. Step 2 of the qPCR was performed and analyzed using the Applied Biosystems 7500 Fast Real-Time PCR machine following the manufacturer's protocol for relative quantification. As described by Bustin [9], expression of the 18S ribosomal subunit was amplified as a housekeeping control gene for qPCR.

Graphing and statistics

The ΔΔCt method was used to calculate qPCR results. Relative values correspond to the mRNA expression of the gene-of-interest/18S Ribosomal subunit and the average relative value corresponds to the mean of all relative values within the same experiment independently determined for each gene-of-interest. All mRNA expression graphs for descriptive studies were displayed as percent of the average relative value instead of percent control because there are no controls in these descriptive studies. Error bars represent standard error. SPSS software was used for Kruskal-Wallis followed by Mann-Whitney tests to determine significance at $p \leq 0.05$. Logarithmic regression analysis for the *TSHβ* standard curve was performed in Microsoft Excel. Linear regression comparing the relationship between expression of TSH subunits in the daily cycle experiments and those of wild red drum was also performed in Microsoft Excel and included Pearson's correlation coefficient (R^2).

Results

Validation of qPCR for TSHβ

To validate the qPCR assay for *TSHβ*, I created a standard curve using known concentrations of *TSHβ* PCR product including concentrations within the range of expected red drum *TSHβ* qPCR results and a range of values far exceeding the expected range (figure 2.1A). The standard curve was linear even

in the range exceeding that of expected values and the R^2 value was near 0.97. Serial dilutions of a red drum calibrator pituitary cDNA diluted parallel to that of a 2fg sample from the standard curve (figure 2.1B). The intra-assay variability measured as CV=6.64. PCR products were sequenced to confirm identity.

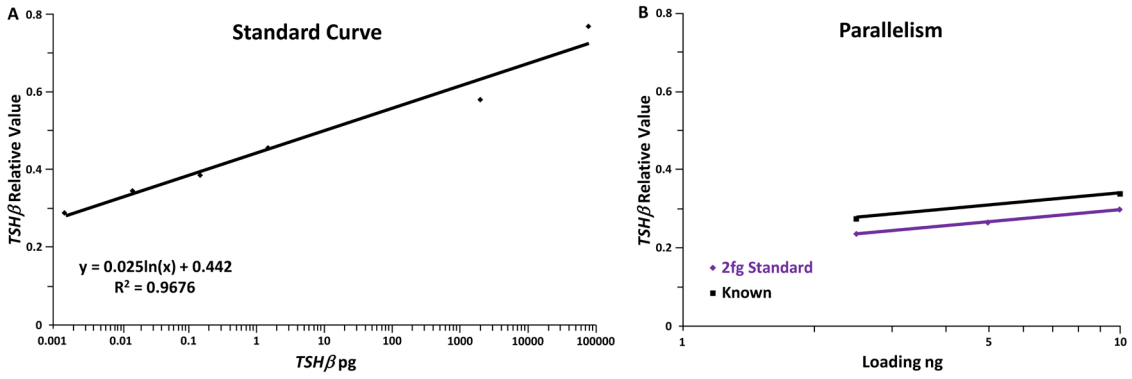

Figure 2.1. qPCR *TSHβ* (A) standard curve and (B) parallelism. (A) Known concentrations of *TSHβ* PCR product are graphed versus the relative value (*TSHβ*/18S) by measurement in the qPCR assay. The regression equation and correlation coefficient are shown. (B) A calibrator pituitary cDNA sample diluted in parallel with the 2fg *TSHβ* qPCR standard.

Cloning and sequencing of red drum Dio2, Dio3, and TSHR

Partial cDNA sequences for each transcript were assembled from overlapping PCR products, subjected to BLAST analysis (NCBI), and confirmed to be *Dio2*, *Dio3*, and *TSHR*. Based on alignments with transcripts of other fish

species, putative sequence elements were determined. The 744bp partial *Dio2* fragment includes sequence from the 5' UTR, the start codon, and the selenocysteine codon (figure 2.2A). The human *Dio2* contains one intron giving rise to alternate splice variants, but these variants do not code for active enzymes [27]. I found no evidence (multiple PCR products) in this predicted exon junction site to support multiple *Dio2* transcripts in red drum. The 437bp partial *Dio3* sequence contains the selenocysteine codon and the stop codon (figure 2.2B). Typically, *Dio3* contains no introns [27], but two transcripts differing only in the 3' UTR were reported in the trout [6]. I did not find multiple *Dio3* 3' UTR PCR products in red drum. The 214bp partial *TSHR* sequence begins in the extracellular domain and brackets the first and second transmembrane domains (figure 2.2C). The red drum *TSHR* fragment is predicted to be within one continuous exon [84], and thus while multiple *TSHR* splice variants might exist in red drum, my PCR would not have and did not detect them. Phylogenetic analysis found that each red drum *Dio2*, *Dio3*, and *TSHR* transcript segregated with respective transcripts from most closely related fish species (figure 2.3).

A **rdDio2 (Partial)**

Nucleotide Sequence

GCAATGTTCGTCCTTTTAAGAAGAGAGATCGAGCTCCGGCGGCC
TCGCGTCTGCGCGCACACGGCGGAACACCTGAAGGGGAACAGA
GCACGCGATACACCTGATACACCTCCCTCACTGTCTGCACCATGC
CTGGAGCAGTCCACGGAGCAGAGCAGAAACTATGAGGGGAGCA
GATGAGACCCACGGCACAGCGTCGCGCGTAATGAAGGGCGGTT
TGGAATAATTCGCTCGTGTAGACGCACAGAGAGAAA**ATG**GGCA
TGGCGAGTGAGGATCTGCTTGTCACACTCCAGATACTACCTGGTT
TCTTTTCGAACTGCCTCTTCCTCGCCCTGTACGACTCCGTGGTTCT
CGTGAAGCGCGTCGTGTCGCTGCTGAGCTGCTCCAGGTCCGCCG
GATCCGGAGAGTGGCGCCGCATGCTGACCTCAGCGGGGCTGCG
CTCCATCTGGAACAGTTTCCTTCTGGACGCCTACAAACAGGTAAA
ACTTGGCTGCGAGGCACCCAACTCCAAACTGGTGAAGGTGCCTG
ATGGCCTTCACTGGAGCAGCAATATCAGTAACATGACTAATGTG
CCACCTGGTGCCAGGATCCGAAATGGAGACGAGTGCCGCCTTCT
GGATTTTGAGTCATCGGATCGCCCTCTGGTGGTCAACTTTGGCTC
AGCCACC**TGA**CCCCCATTCATCAGCCACCTGCCAGCTTTCCGGC
AGTTGGTGGAGGACTTCAGTGATGTGGCTGATTTTCTGT

Amino Acid Sequence

mgmasedllvtlqilpgffsnclflalydsvvlvkrvvsllscsrsagsgewrrmltsag
lrsiwnsflldaykqvklgceapnsklvkvpdglhwssnisnmtnvppgarirngd
ecrlldfessdrplvvnfgsatuppfishlpafrqlvedfsdvadfl

B **rdDio3 (Partial)**

Nucleotide Sequence

ACTTTGGCAGCTGCACC**TGA**CCACCGTTCATGACGCGCCTGGCG
GCGTTTCAGCGCGTCGTGAGCCAGTACGCAGACATTGCGGACTT
TTTAGTTGTATATATCGAGGAGGCGCACCCGTCGGACGGCTGGG
TGAGCTCGGACGCTCCGTATCAGATCCCCAAGCACCGCTGTTTG
GAGGACAGACTGAGAGCAGCTCAGCTGATGCTCACCGAGGTGC
CGGGGAGCAACGTGGTGGTGGATAATATGGACAACTCATCCAA
CGCCGCGTACGGAGCCTACTTTGAGAGACTTTACATCGTGAGGG
ATGAGAGAGTGGTGTACCAGGGGGGCAGAGGTCCAGAGGGAT
ACCGGATTTCCGAGCTTAGAAACTGGCTGGAGCAATACAGGAAC
GATCTGGTGAATTCCAAAAGAGCGGTGCTCCATGTG**TAG**TGAA
AA

Amino Acid Sequence

fgsctuppfmtrlaafqrvvsqyadiadflvvyieeahpsdgwvssdapyqipkhr
cledrlraaqlmltevpgsnvvvdnmdnssnaaygayferlyivrdervvyqggrg
pegyriselrnwleqyrndlvnskravlhv

C **rdTSHR (Partial)**

Nucleotide Sequence

ATCATGGGTTTTGGCTTCCTGCGAGTGTCGGTGTGGTTTGTGAGT
CTGCTGGCTATTCTGGGAAATGTGGTGGTGCTTCTGGTGCTGCTA
ACCAGCCACTACAAGCTCTCGGTCTCGCGCTTCCTCATGTGTCAC
CTGGCCTTTGCAGATCTTTGCATGGGGATTTATCTGCTGCTTATC
GCCTCTGTAGACCTCCACACACGAGCAGAGTATT

Amino Acid Sequence

imgfgflrvsvwfvsllailgnvvvllvlltshyklsvsrflmchlafadlcmgiylliasv
dlhtraey

Figure 2.2. Partial sequences of red drum (A) deiodinase 2, (B) deiodinase 3, and (C) thyrotropin receptor. cDNA nucleotides are shown in capital letters with start, selenocysteine, and stop codons in bold. The proposed translated amino acid sequence is shown in lowercase below the nucleotide sequence.

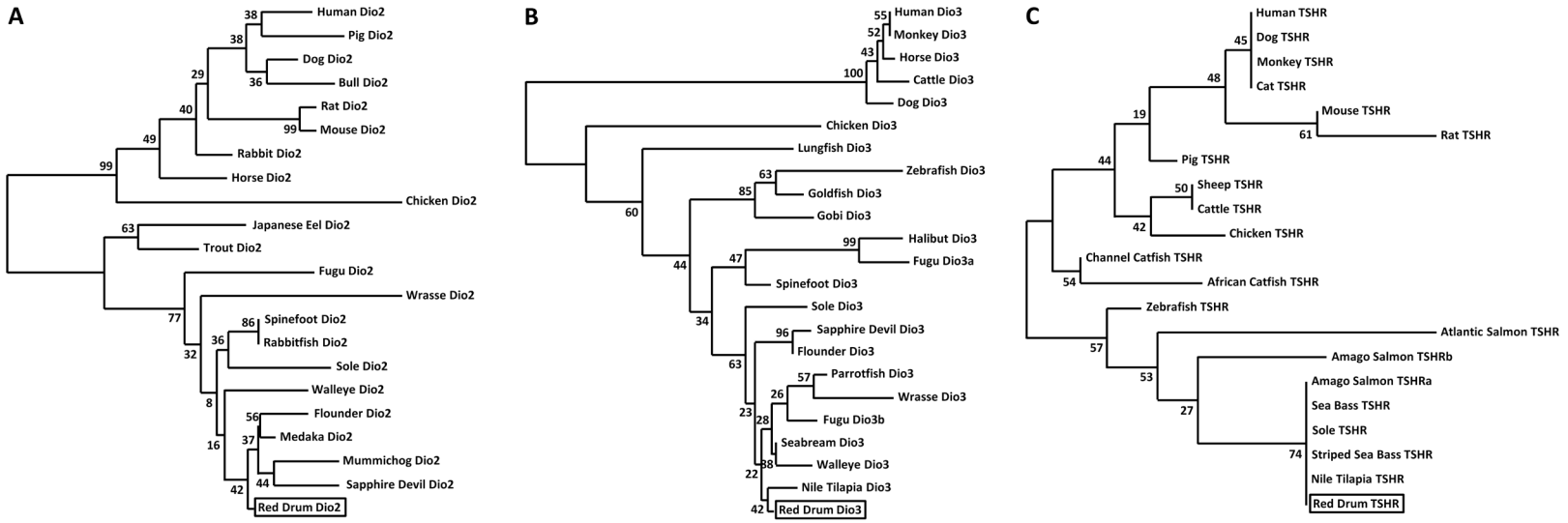

Figure 2.3. Phylogenetic tree of red drum (A) deiodinase 2, (B) deiodinase 3, and (C) thyrotropin receptor. The red drum sequence of interest is boxed within the phylogenetic tree. Numbers at branch points are bootstrap values.

Tissue specific expression of TSHβ, GSUα, Dio2, Dio3, and TSHR in red drum

TSHR was expressed in the lower jaw (figure 2.4). In red drum *TSHβ, GSUα*, and *TSHR* were co-expressed in the pituitary gland (figures 2.6 and 2.8), the gonad (figure 2.14), and the intestine (figure 2.4). *Dio2* and *Dio3* are expressed in the liver and pituitary of red drum (figure 2.5).

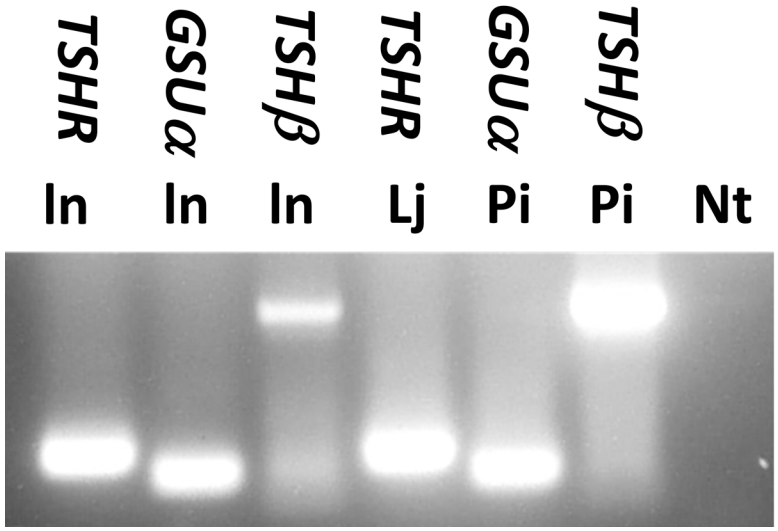

Figure 2.4. Red drum ectopic intestinal *TSHβ, GSUα,* and *TSHR* mRNA expression. The expression of *TSHβ, GSUα,* and *TSHR* is demonstrated by RT-PCR followed by gel electrophoresis and visualized under UV light. Nt=no template control for PCR contamination, Pi=pituitary for *TSHβ and GSUα* positive control, Lj=lower jaw (thyroid gland) for *TSHR* positive control, In=intestine.

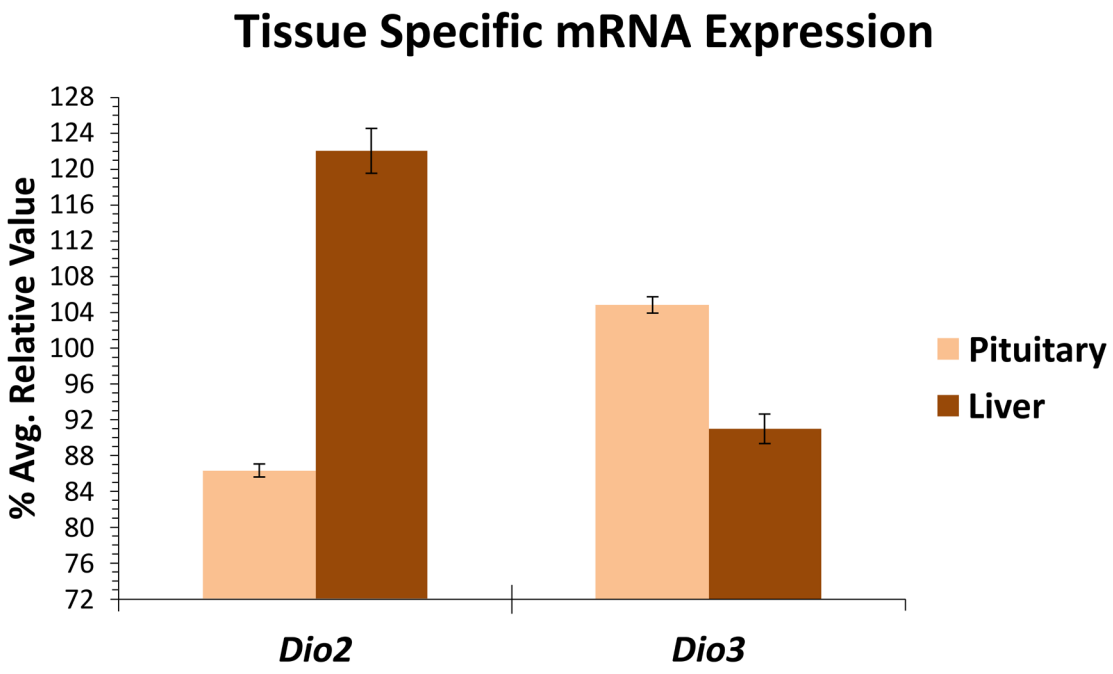

Figure 2.5. Pituitary and liver mRNA expression of *Dio2* and *Dio3* in juvenile red drum. The relative expression of *Dio2* and *Dio3* is graphed representing the transcripts in the pituitary (in light pink) versus the liver (in dark tan). Sample size was 24 per group.

Basal T_4 and gene expression over 24h

I performed two cycle experiments. In the first cycle experiment T_4 followed an expected red drum pattern, rising in the photophase and then falling during the scotophase. The pituitary expression of *TSH*β (figure 2.6A), *GSU*α (figure 2.6B), *Dio2* (figure 2.7A), *Dio3* (figure 2.7B), and *TSHR* (figure 2.8) were compared to circulating T_4 levels (graphed in the background of each gene expression graph) in animals sampled every 4h in the first cycle experiment. In this first cycle experiment *TSH*β*, GSU*α*,* and *Dio3* (figures 2.6 and 2.7B) fell during the photophase and then rose in the scotophase, displaying the inverse of the T_4 cycle. In the first cycle experiment, *Dio2* expression peaked as circulating T_4 reached its acrophase and bathyphase (figure 2.7A). *TSHR* expression in this first cycle experiment peaked during the acrophase of the T_4 cycle (figure 2.8).

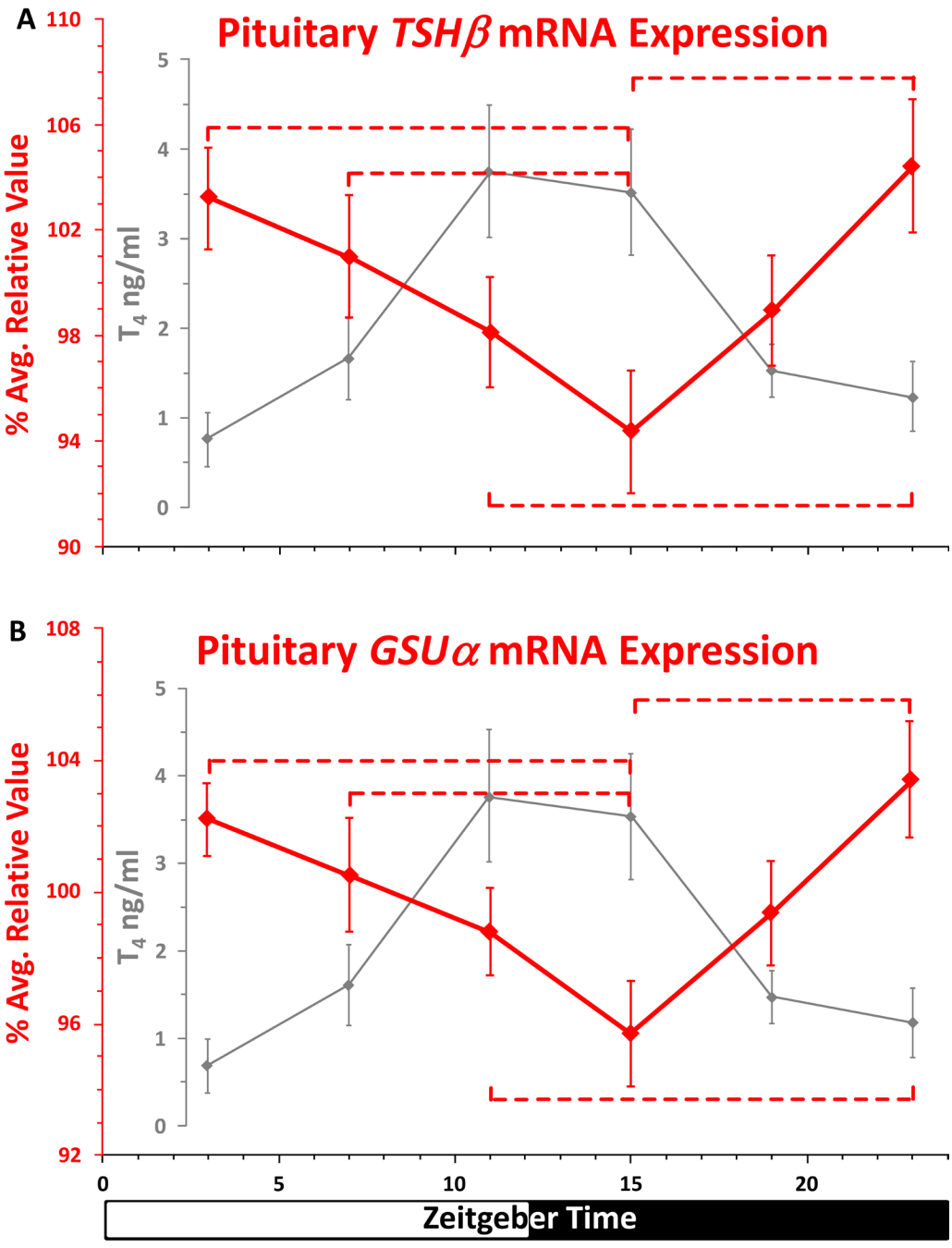

Figure 2.6. Daily cycle of (A) *TSHβ* and (B) *GSUα* mRNA expression in the red drum pituitary (first cycle experiment). The daily changes in lab red drum pituitary (A) *TSHβ* and (B) *GSUα* expression are shown in the foreground in red while circulating T_4 levels in the same animals are shown in grey in the background. The photophase is shown as a solid white box next to the solid black box representing the scotophase below each graph. Significant differences ($p \leq 0.05$) are shown as dashed lines connecting data points. Sample size was 4 replicate pools per time point.

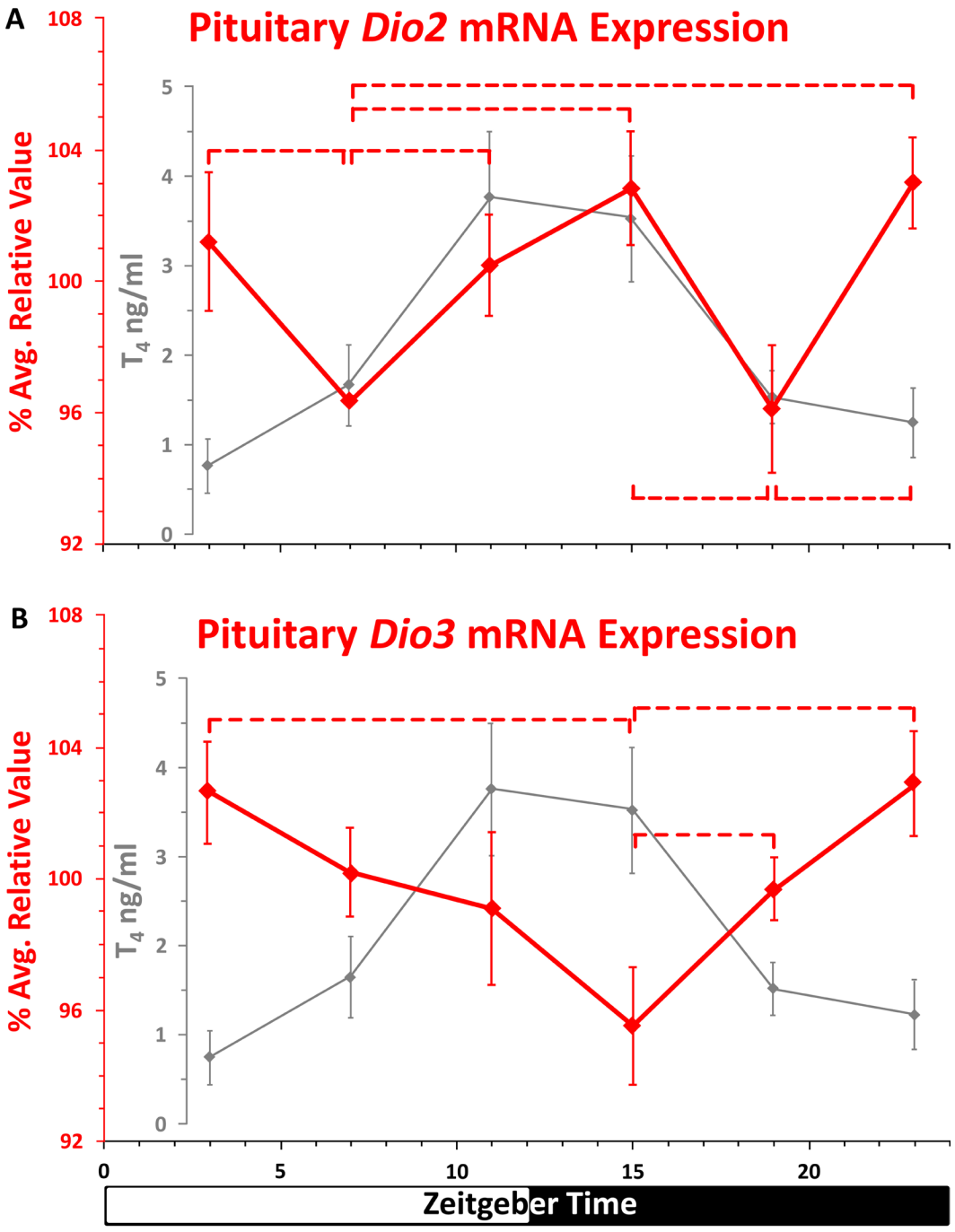

Figure 2.7. Daily cycle of (A) *Dio2* and (B) *Dio3* mRNA expression in the red drum pituitary (first cycle experiment). See figure caption 2.6 for axis details.

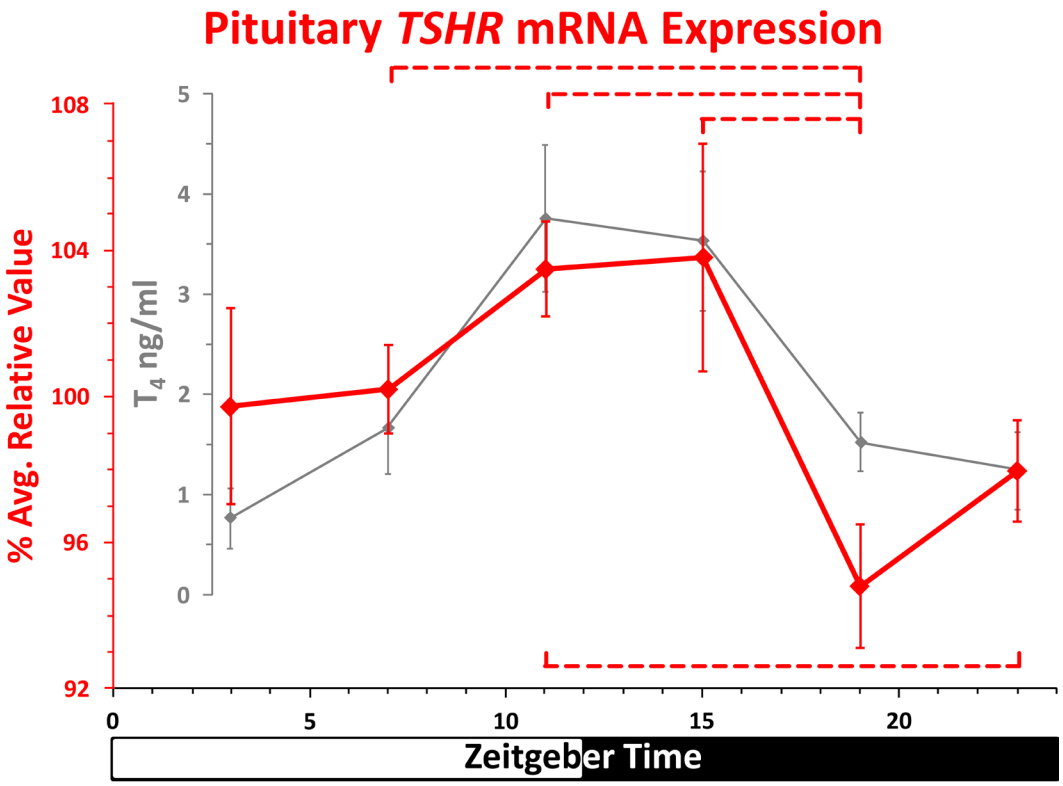

Figure 2.8. Daily cycle of *TSHR* mRNA expression in the red drum pituitary (first cycle experiment). See figure caption 2.6 for axis details.

The first time point in the second cycle T_4 data were significantly and abnormally high. After this time point, the T_4 cycle in this second experiment returned to the standard red drum cycle matching previous cycles showing a T_4 increase continuing from shortly before lights on to a T_4 peak slightly after lights off. The pituitary expression of *TSHβ* (figure 2.9A), *GSUα* (figure 2.9B), and *Dio3* (figure 2.10B) in the 2nd cycle experiment all rose in the photophase, fell early and late in the scotophase, with a peak during mid scotophase. The variance of *Dio2* (figure 2.10A) expression in the second cycle experiment was very high, and as such, no pattern can be elucidated from these data (figure 2.10A). Insufficient RNA was available from the second cycle experiment to measure *TSHR* expression. In both cycle experiments the expression of *TSHβ, GSUα,* and *Dio3* was reciprocal to T_4.

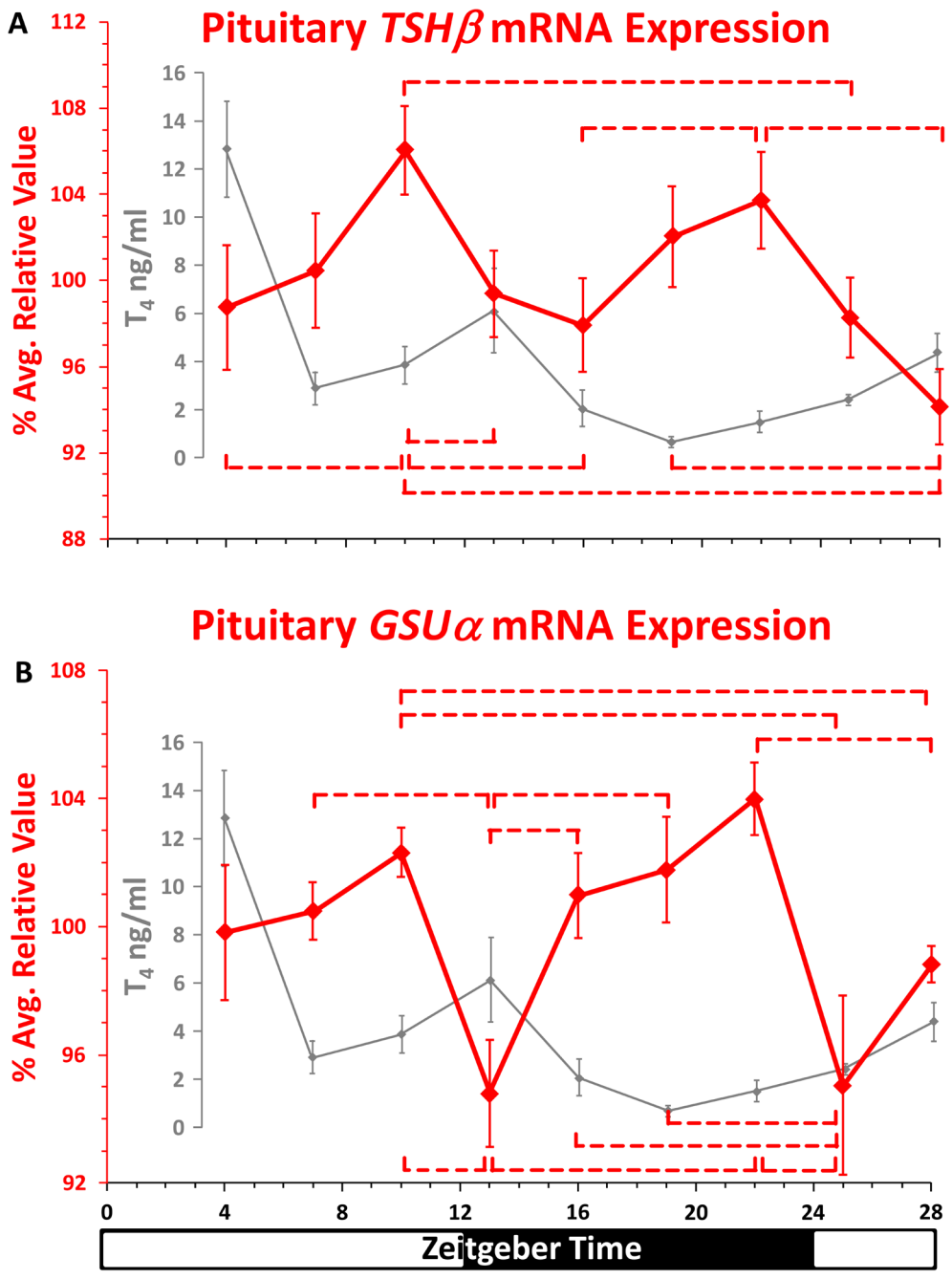

Figure 2.9. Daily cycle of (A) *TSHβ* and (B) *GSUα* mRNA expression in the red drum pituitary (second cycle experiment). See figure caption 2.6 for axis details with the exception that the sample size in this experiment was 8 per time point.

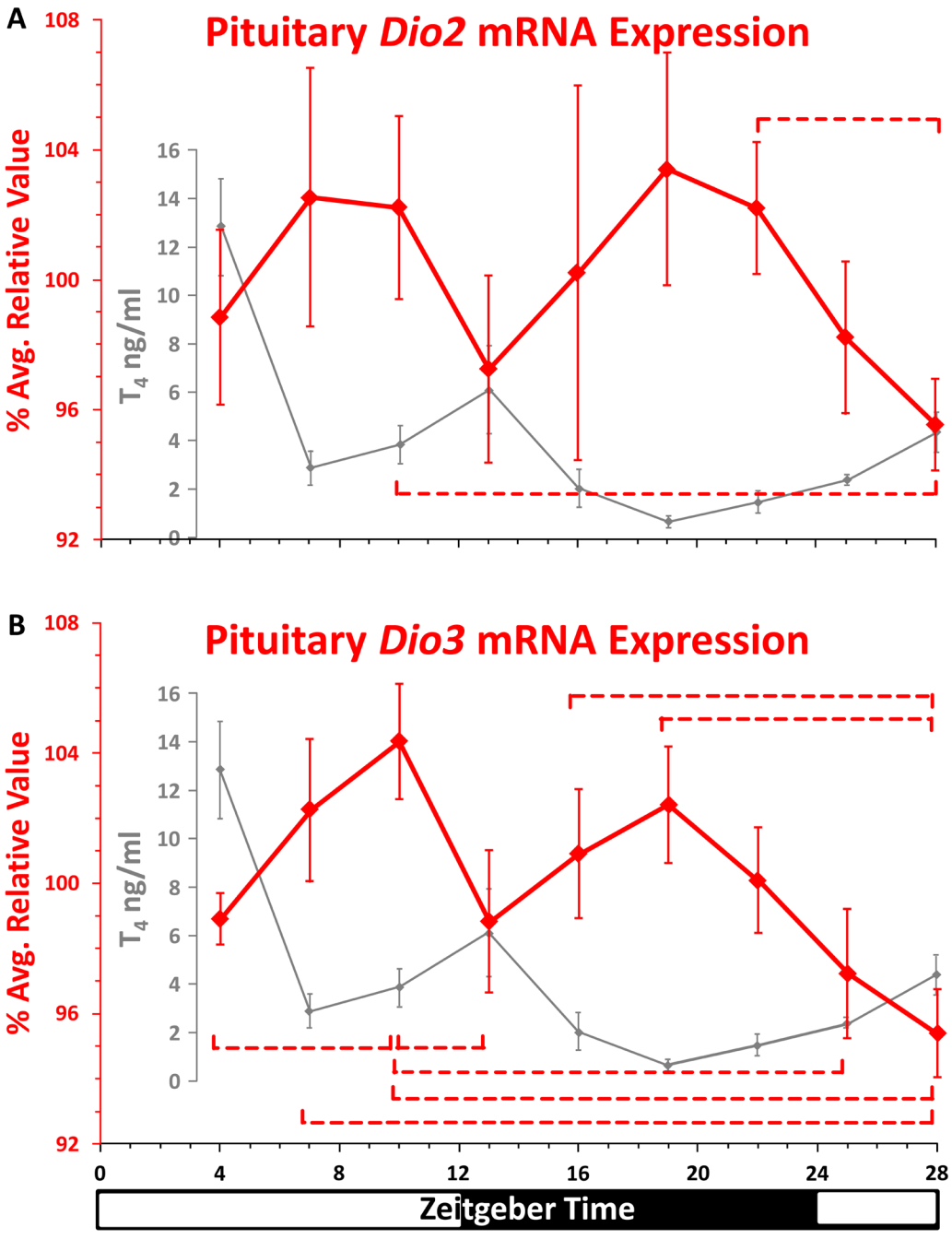

Figure 2.10. Daily cycle of (A) *Dio2* and (B) *Dio3* mRNA expression in the red drum pituitary (second cycle experiment). See figure caption 2.6 for axis details with the exception that the sample size in this experiment was 8 per time point.

TSHβ, GSUα, Dio2, Dio3, and TSHR in lab versus wild red drum

TSHβ expression was significantly higher (11.7%) in juvenile lab animals than in adult wild red drum while pituitary *TSHR* expression was significantly higher (8.4%) in wild fish than lab fish (figure 2.11). Pituitary expression of *GSUα*, *Dio2*, and *Dio3* did not differ between lab and wild populations (figure 2.11). Pituitary expression did not differ between sexes for any of the measured transcripts (figures 2.12). Pituitary expression of *TSHβ*, *GSUα*, and *Dio2* in wild animals did not vary between months (figure 2.13). In wild animals pituitary *Dio3* expression was significantly lower in the July and August samples than in the April samples (figure 2.13). In April and July wild pituitary *TSHR* transcripts were not significantly different from one another, but all other comparisons of wild pituitary *TSHR* expression were significantly different (figure 2.13). Linear regression analysis showed that the expression of TSH subunits in juvenile lab animals (data from the daily cycle experiments) varied together with a R^2 value near 0.87 while the *GSUα* and *TSHβ* subunits did not vary together with such a strong relationship in wild animals (figure 2.14).

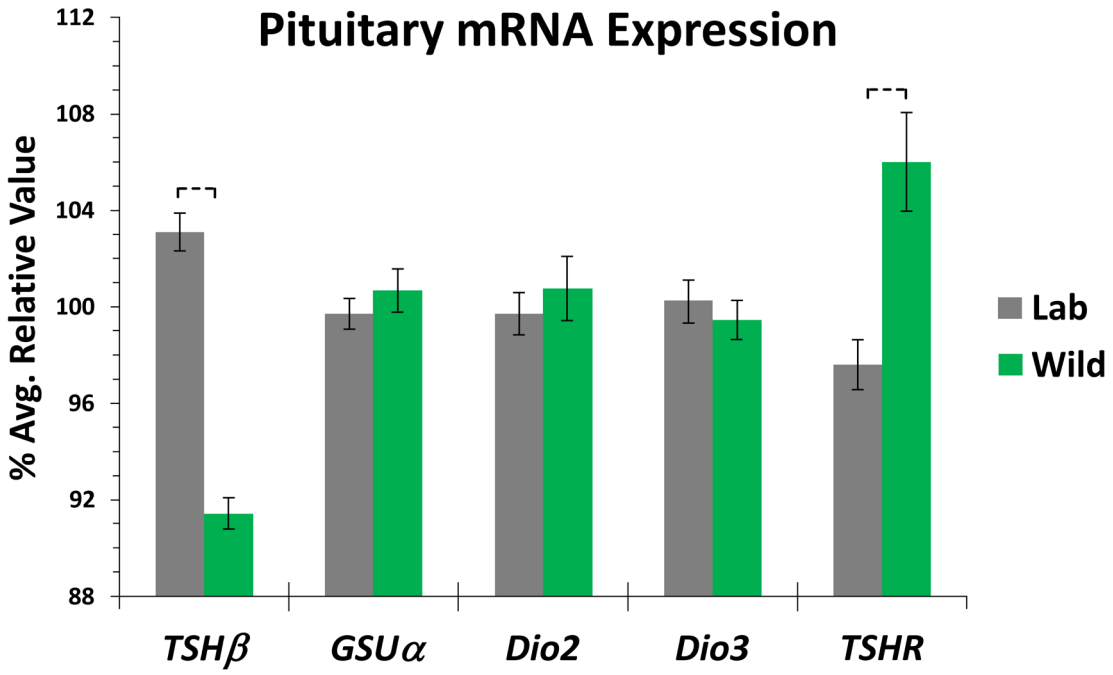

Figure 2.11. Lab versus wild *TSHβ, GSUα, Dio2, Dio3*, and *TSHR* mRNA expression in the red drum pituitary. Expression is graphed representing mRNA expression in juvenile lab red drum (in grey) versus in adult wild red drum (in green). Significant differences ($p \leq 0.05$) are shown as dashed lines connecting groups. Sample size was 45 for lab pituitaries and 24 for wild pituitaries.

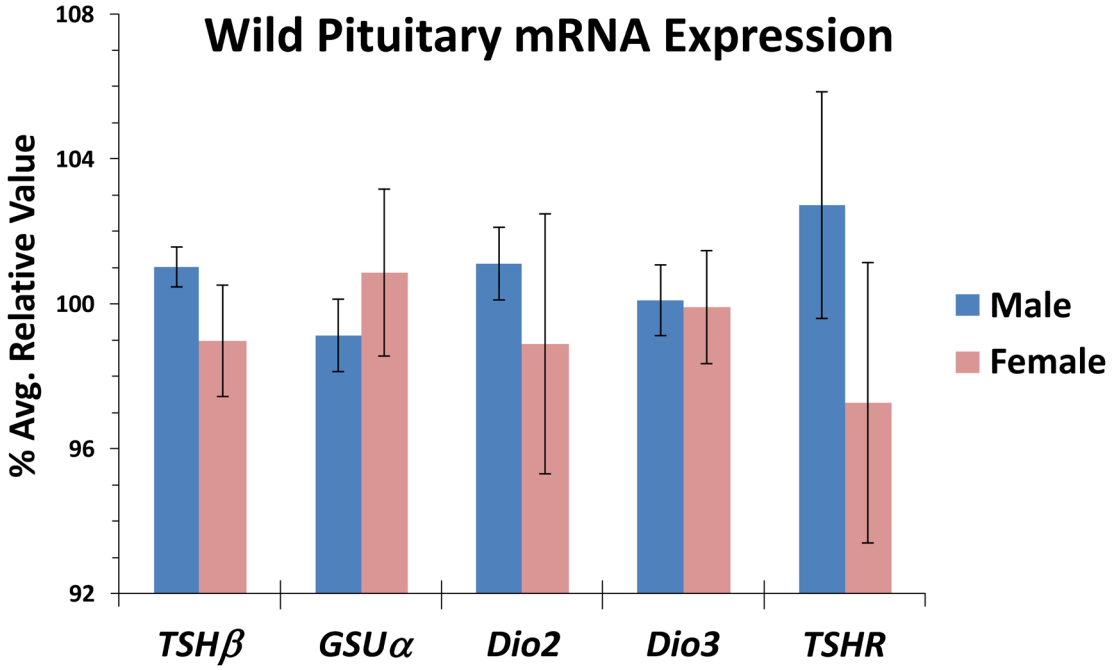

Figure 2.12. Male versus female *TSHβ, GSUα, Dio2, Dio3*, and *TSHR* mRNA expression in wild red drum pituitaries. Expression of each transcript in wild red drum is graphed representing the transcripts in males (in blue) versus females (in pink). There were no significant differences. Sample size was 3 per sex per gene product.

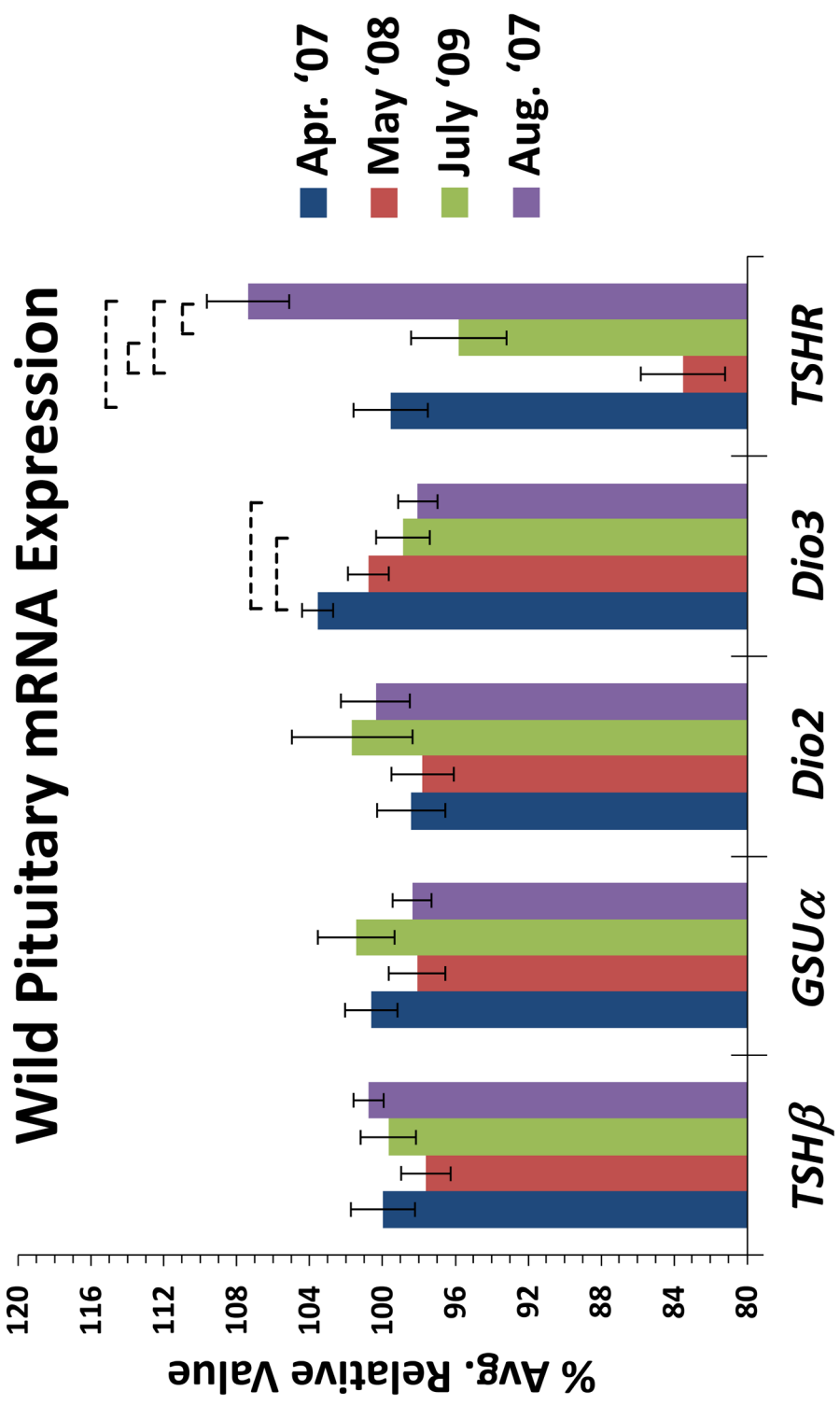

Figure 2.13. *TSHβ*, *GSUα*, *Dio2*, *Dio3*, and *TSHR* mRNA expression in wild red drum pituitaries over time. Expression in wild red drum pituitaries graphed by month sampled. The figure legend assigns colors to the months sampled. Significant differences ($p \leq 0.05$) are shown as dashed lines connecting groups. Sample size was 6 per month per transcript.

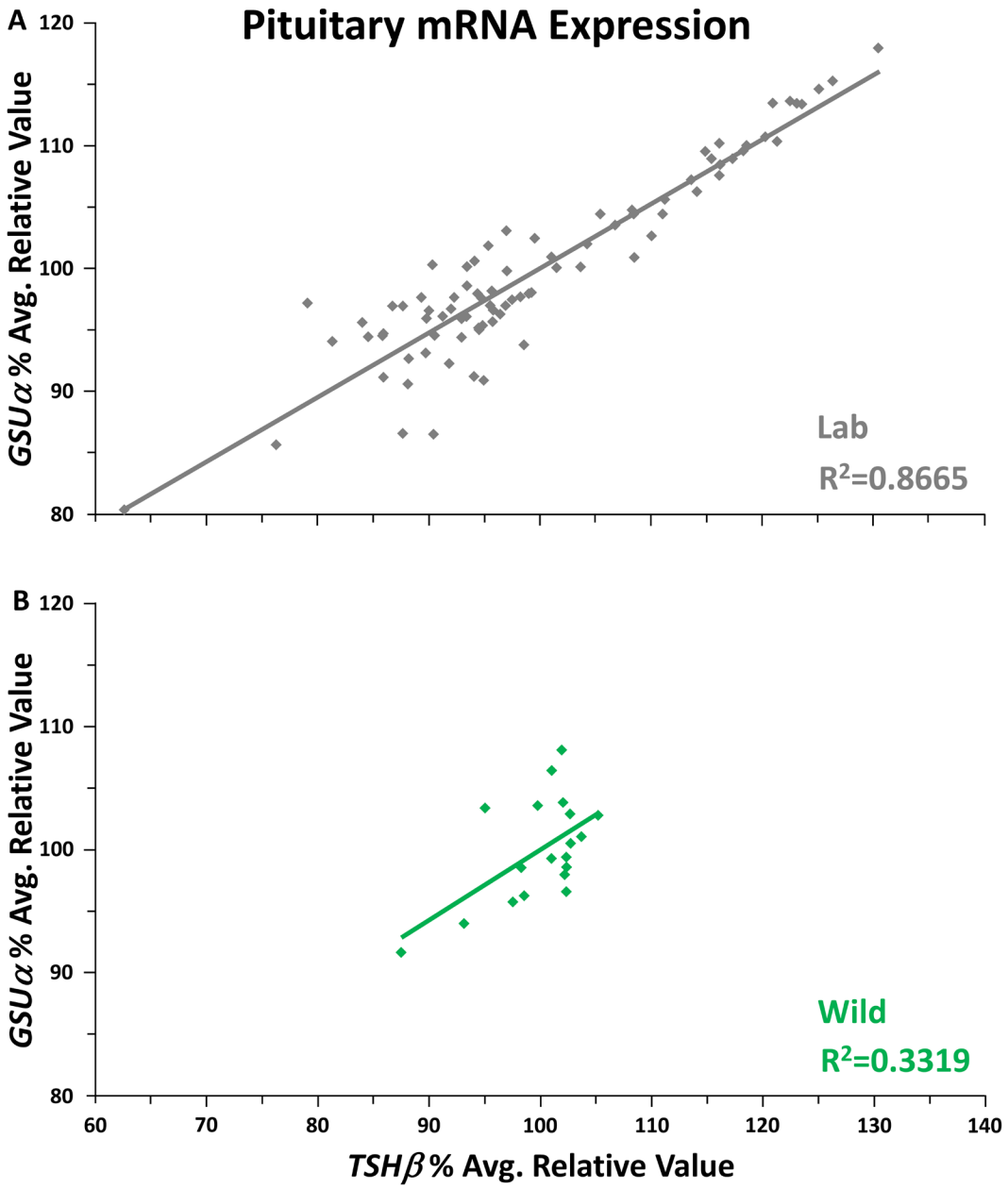

Figure 2.14. Pituitary correlation between TSH subunits in (A) lab and (B) wild red drum. Simple linear regression was used to predict the relationship between *TSHβ* and *GSUα* pituitary mRNA expression in (A) juvenile lab animals (in grey) and (B) adult wild red drum (in green). A correlation coefficient (R^2) is included with each plot. Eighty-four and nineteen comparisons were available from lab and wild animals, respectively.

Gonadal expression of TSHβ, GSUα, and TSHR in wild red drum April, May, July, August

Testicular *TSHβ* expression was significantly higher in May than August samples (figure 2.15). Testicular *GSUα* expression did not differ among any months sampled (figure 2.15). Testicular *TSHR* expression was higher in August than April samples (figure 2.15). Ovarian *TSHβ* expression was significantly higher in April than May samples (figure 2.15). Ovarian expression of *GSUα* did not differ between May and August samples, but all other comparisons of ovarian *GSUα* expression were significantly different (figure 2.15). Ovarian expression of *TSHR* was significantly higher in August samples than April, May, and July samples (figure 2.15). Overall, the expression of *TSHR* was 11.1% significantly higher in the testis than ovary (figure 2.15).

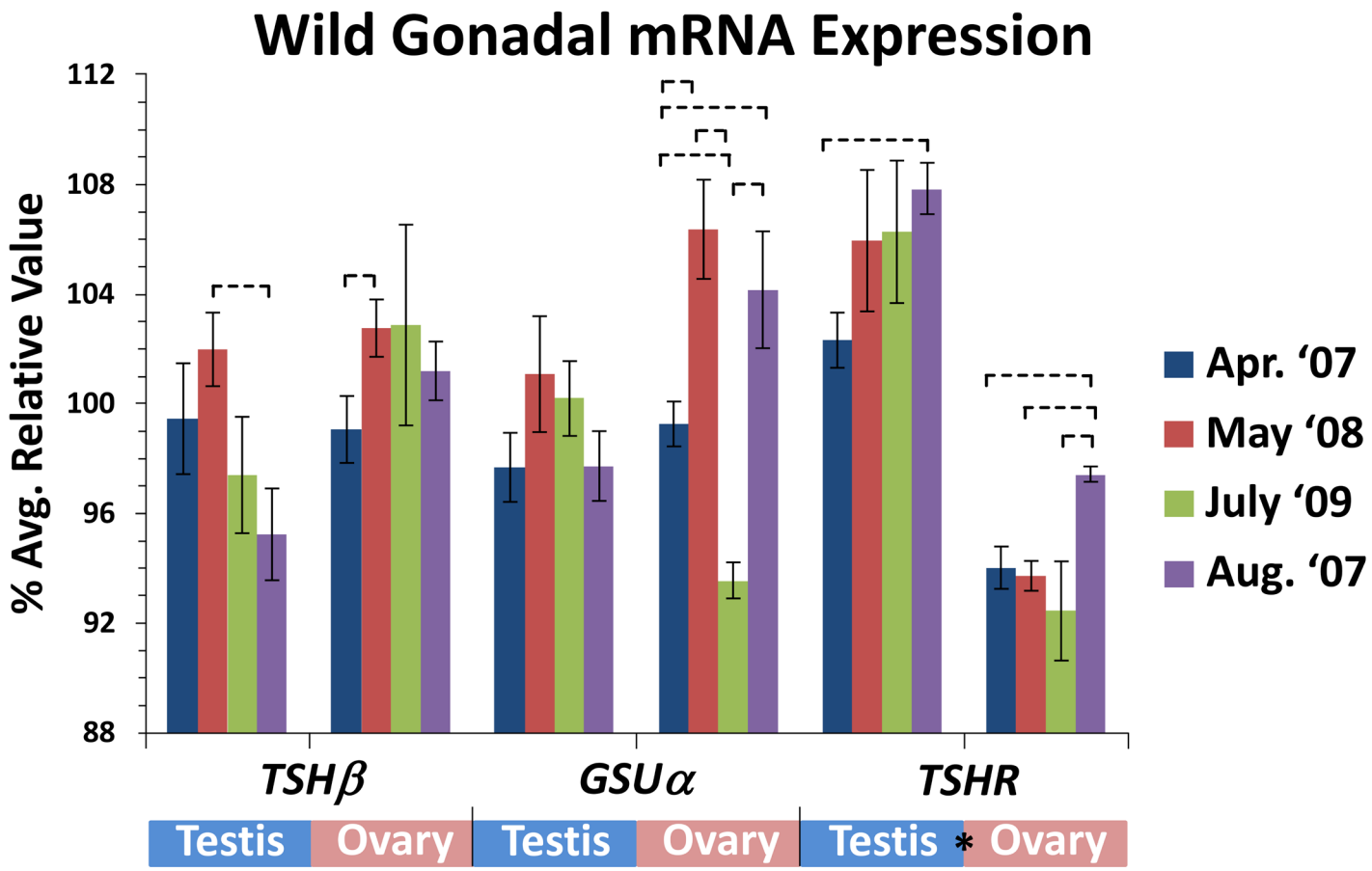

Figure 2.15. Gonadal *TSHβ, GSUα,* and *TSHR* mRNA expression in wild red drum. Expression in wild red drum gonads is graphed by transcript and month. Males are indicated by blue labels while females are indicated by pink labels in the axis title. Significant differences ($p \leq 0.05$) are shown as dashed lines connecting groups. An overall significant difference ($p \leq 0.05$) between *TSHR* expression in the ovary and testis is indicated by an asterisk in the horizontal axis title. Sample size was 3 per gonad per month per transcript.

Discussion

In this study I have developed new techniques for quantification of the expression of genes for pituitary proteins that for the first time have enabled the simultaneous characterization of daily changes in a suite of proteins proposed to be important in the central regulation of thyroid function in fish. This enabled me to compare the relationship among *TSHβ, GSUα, Dio2, Dio3*, and *TSHR* and circulating T_4 in a fish species already known to exhibit dynamic daily rhythms of T_4 to test the hypothesis that negative feedback of circulating hormones is important in the regulation of expression of pituitary TSH. These techniques also enabled me to measure the expression of these gene products in other ectopic locations. To address these objectives I developed qPCR to measure thyroid-related transcripts in red drum.

Bustin [9] concluded that qPCR is the method of choice for experiments that require sensitive, specific, and reproducible quantification of mRNA. Additionally, the qPCR method is safer than traditional radioactive blotting techniques [9]. qPCR has been used in previous teleost studies to measure mRNA expression for *TSHβ* and *GSUα* but rarely in *in vivo* studies with multiple replicates. A distinct advantage of the sensitivity of qPCR is the ability to obtain sufficient replication without pooling tissue from multiple animals, thus more accurately reflecting biological variation while utilizing fewer animals per experimental treatment. I developed new qPCR assays that could measure

multiple (5-6) gene products from single pituitary glands taken from fish as small as 20g. These techniques allowed me compare the simultaneous expression of multiple thyroid-related gene products in individual pituitary glands throughout a daily cycle in juvenile animals. Our *TSHβ* qPCR assay was chosen as a representative assay to examine the dynamics of qPCR from red drum samples. Techniques typically used to validate radioimmunoassay (RIA), such as linearity of a standard curve and parallelism, also suggested that the qPCR *TSHβ* assay was valid. PCR products confirmed the identity of target genes. I was then able to use these qPCR techniques to examine changes in thyroid-related mRNA expression throughout a daily cycle to begin addressing the hypothesis that TSH drives a daily elevation in circulating T_4 that in turn inhibits TSH production through negative feedback.

Leiner et al. [47] described a robust daily rhythm of circulating T_4 in red drum and a less robust rhythm of T_3. I replicated this T_4 cycle by observing that T_4 rises during the day and falls at night in two additional cycle experiments. Leiner and MacKenzie [49] proposed that a centrally-driven circadian rhythm of circulating T_4 in red drum was controlled by TSH secretion during the day, driving increasing circulating T_4 levels in the morning. In this model T_4 negative feedback then inhibited TSH production during the afternoon. Given that the red drum cycle was hypothesized to be regulated in part by T_4 feedback, deiodinase expression would be required in this model to create a cycling pituitary supply of T_3 for receptor binding and subsequent TSH regulation. My study was aimed at

testing this model by evaluating the mRNA expression of the TSH subunits and deiodinases 2 and 3 concurrently with circulating T_4.

The T_4 cycle in the first experiment closely matched previous cycles observed in our laboratory. The second cycle experiment also replicated previous cycle experiments but with one difference. There was a transient, anomalous peak in T_4 observed in the morning of our second cycle experiment. Following this one anomalous point, the T_4 cycle returned to that typical of previous red drum experiments, such as reported by Cohn et al. [15]. This anomalous peak, which has not been observed before in over 20 cycle experiments, was most likely a result of a novel diet including iodine supplementation, used to treat increasing mortality in captive red drum. The iodine-supplemented food was fed 2h prior to the anomalous sampling time, and this feeding treatment was the only substantive difference between this experiment and previous cycle experiments lacking this T_4 surge. No surge was observed at the comparable time the next day when the fish were not fed until after the experiment was over. In humans TH secretion is inhibited by acute iodine, but in the trout acute iodine treatment can increase circulating T_4 [21]. If dietary iodine supplementation is indeed capable of eliciting such a dramatic, yet transient alterations in circulating thyroid hormone levels in red drum, further study of dietary iodine effects on thyroid function in fish may be a productive pathway in providing a better understanding of the regulation of circulating thyroxine cycles. Studies are currently underway in our laboratory to study the

effects of low dose dietary iodine on thyroid function in red drum. Regardless of the cause, this anomalous point provided a unique opportunity to address the hypothesis that pituitary expression of TSH subunits and deiodinases was being driven by circulating T_4 levels. A disrupted T_4 cycle would be expected to disrupt patterns of pituitary expression of proteins regulated by circulating T_4 feedback.

TSH, including the mRNA for both TSH subunits, is well known in mammalian species to be under the control of hypothalamic stimulation and negative feedback regulation by circulating THs [11, 25, 80]. TSH daily cycles are well documented in mammals including mice and humans, where TSH appears to drive downstream cyclic T_4 or T_3 blood levels [10, 73]. Brabant et al. [5] suggested that cycles of TSH secretion could regulate all aspects of thyroid activation. In fish, not only is there no information on the cyclic nature of pituitary TSH production, but the overall significance of TSH in the regulation of thyroid function has been called into question because of its predominantly inhibitory control and the relatively low levels of its pituitary expression [21, 55]. Glycoprotein gene regulation, secretory production and storage, and secretion can occur within six hours [72]. In common carp previtellogenic females and spermiating males, in response to salmon gonadotropin releasing hormone injection, pituitary luteinizing hormone (a glycoprotein hormone similar to TSH) β-subunit mRNA expression and circulating LH increased within 6h and persisted for 24h, demonstrating that rapid increases in glycoprotein hormone mRNA expression can be associated with rapid subsequent increases in

circulating hormone [38]. If TSH responds similarly, then a 6h delay between expression and secretion could be reasonably expected. Based on mammalian studies, daily cycles of circulating TSH should precede TH release from the thyroid gland by 1.5-4h [10, 73], although TSH can stimulate circulating T_4 as rapidly as 30 minutes [60]. Robust increases in *TSH*β and *GSU*α mRNA expression in my first cycle experiment which preceded T_4 by 6-12h, thus provided a reasonable time delay for TSH translation, secretion, and stimulation of the thyroid. *TSH*β and *GSU*α were also expressed in an inverse relationship to circulating T_4, suggesting that TSH-stimulated T_4 elevations subsequently inhibited TSH expression later in the cycle through negative feedback. In the second cycle experiment *TSH*β and *GSU*α expression did not exhibit the same daily pattern observed in the previous experiment. Interestingly however, the expression of TSH subunits in this experiment was, as seen in the first experiment, inversely related to circulating T_4 in the anomalous cycle. In the second cycle experiment the anomalous T_4 surge likely shifted the temporal expression of *TSH*β and *GSU*α to a bimodal cycle, yet both TSH subunits were still clearly expressed in an inverse relationship to circulating T_4. This reciprocal pattern of expression to circulating hormone is consistent with negative feedback regulation of TSH subunit expression by circulating T_4. These data do not, however, support negative feedback as the sole regulator of TSH expression in red drum. If T_4 negative feedback were the sole regulator of pituitary TSH expression, then we would expect the 10h time point in the second cycle

experiment to exhibit the lowest blood T_4 value given its high *TSH*β and *GSU*α expression. These data support the contention by Leiner and MacKenzie [49] that the daily T_4 cycle is due to interplay between stimulation of the hypothalamo-pituitary-thyroid axis by a circadian oscillator early in the cycle followed by central inhibition of the axis late in the cycle.

In mammals, T_3 for negative feedback on TSH expression is generated in the pituitary by Dio2 deiodination of T_4 [3, 63] thus making Dio2 critical for TH actions in the pituitary. Further studies have shown that Dio2 cycles in the rat pituitary inverse to the TSH cycle, thereby Dio2 in the rat controls T_3 availability for feedback [54, 62]. The physiological regulation of *Dio2* mRNA expression in the fish pituitary has not been examined. In my study I found, as expected, that *Dio2* was expressed in both the liver and pituitary. I also found in the red drum that while pituitary *Dio2* expression was readily measurable, signifying active conversion of T_4 to T_3 for TH action in the pituitary, the expression of *Dio2* varied widely in both cycle experiments. In the first cycle experiment, a *Dio2* acrophase coincided with the bathyphase of the *TSH*β and *GSU*α cycles. At this time point T_3 production by Dio2 should be needed for negative feedback, which should be maximal. In the second cycle experiment *Dio2* variability was high making it impossible to establish a clear relationship between *Dio2*, TSH subunits, and circulating T_4. Given these data, *Dio2* does not appear to be regulated in a fashion singlehandedly responsible for establishing negative feedback tone in the control of the daily cycle of TSH in red drum. It is conceivable that in the red

drum a constitutive expression of *Dio2* is sufficient for providing sufficient T_3 for negative feedback function within the pituitary.

Mammalian thyroid function is thought to be regulated by the hypothalamic-pituitary-thyroid axis to maintain a T_4 set-point with deiodinase activity present in peripheral tissues, but not the rate limiting factor in hormone activation for peripheral actions [21]. In this context, the function of Dio3 in mammalian pituitary cells is far less studied than Dio2 [24]. As recently as 2002, mammalian researchers thought Dio3 to be expressed in most tissues but not in the pituitary [3, 41, 42]. However, *Dio3* mRNA has been recently discovered in the mouse pituitary and found to be regulated by T_3 [2]. In fish the balance between ORD and IRD in peripheral tissues is thought to regulate thyroid function by controlling a T_3 set-point [21]. In fish in this context, much interest has been focused on the regulation of ORD without emphasis on IRD [21]. Recent studies though have begun to focus on IRD. For example, in the channel catfish, seasonal cycles of THs are associated with major seasonal alterations in hepatic IRD activity and not hepatic ORD [53]. Likewise, in rainbow trout, liver *Dio3* mRNA expression is regulated by T_3 but not by T_4, suggesting that peripheral mechanisms in this fish are protecting a T_3 set-point [6]. However, the physiological regulation of *Dio3* mRNA expression in the fish pituitary has not been examined. In my study I found that red drum *Dio3* was expressed in the liver and strongly in the pituitary, suggesting that it may play a role in the regulation of fish TSH cycles. The pituitary expression of *Dio3* exhibited

intriguing and novel results in my study. While *Dio2* expression in the pituitary did not appear to be cyclic, the expression of *Dio3* showed a pronounced cycle. In the first cycle experiment, the expression of *Dio3* cycled coincident with *TSH*β and *GSU*α suggesting that the Dio3 deactivation of intracellular T_3 and T_4 removes inhibitory regulation on TSH, allowing TSH expression to increase. In the second cycle experiment *Dio3* cycled once again with the same coincident relationship to TSH, even in the face of the anomalous T_4 cycle. In both cycle experiments *Dio3* cycled with a similar inverse relationship to circulating T_4 even in the second cycle experiment where the anomalous T_4 surge likely shifted the temporal expression of *Dio3* to a bimodal cycle. From these data I propose that Dio3 plays a critical role in the cyclic expression of TSH and that its expression is regulated by circulating T_4. A current working hypothesis is that as T_4 increases it activates a decrease in Dio3 expression, likely preventing the destruction of intracellular T_4 and T_3 in thyrotrophs, thus allowing for T_3 production by constitutively expressed Dio2 to exert negative feedback regulation of TSH. Conversely, as T_4 decreases, Dio3 increases, catabolizing intracellular T_4 and T_3, and thus decreasing pituitary T_3, resulting in a release of TSH from negative feedback and promoting subsequent TSH increase during the photophase. TSH would then be available to drive increased T_4 secretion in the next cycle. The reciprocal relationship between T_4 and *Dio3* expression exemplifies the importance of T_4 inactivation in the pituitary permissively contributing to the regulation of the TSH cycle. This cycle of *Dio3* expression in

the pituitary is novel. This discovery of significant cyclic expression of *Dio3* in the pituitary helps underscore the necessity of a closer examination of this catabolic enzyme. Dio3 may play a more important role in the fish pituitary in comparison to mammals in protecting the precisely-regulated TSH negative feedback system from circulating T_3 inhibition, as fish generally exhibit much higher circulating T_3 levels than mammals.

Using my TSH subunit qPCR assays I was able, for only to second time, to measure the mRNA expression of *TSH*β and *GSU*α as they change over the course of the day in a teleost fish. The previous daily cycle of *TSH*β and *GSU*α mRNA expression was reported from our lab using the radioactive dot blotting technique [15]. Although I reanalyzed the same samples from this previous experiment, my qPCR assay did not yield the same results as the dot blotting technique. The dot blot data showed *TSH*β and *GSU*α expression peaking 4h after circulating T_4 peaked. The opposite result would be expected if TSH expression results in relatively rapid TSH secretion to drive T_4 release. While the cycle from the dot blot did not follow a simple physiological explanation, my qPCR results are more easily explained by physiological processes that had been previously hypothesized to contribute to the regulation of TSH in the red drum pituitary [49]. Leiner and MacKenzie [49] proposed that TSH production drives T_4 release early in the cycle. My qPCR data support this proposal as demonstrated by TSH expression leading to circulating T_4. Leiner and MacKenzie [49] also proposed that T_4 negative feedback inhibited TSH

production to regulate the downward phase of the T_4 cycle. My qPCR data also supported this proposal by showing that TSH expression is reciprocal to T_4. The dot blots were designed to hybridize near the 3' end of both *TSH*β and *GSU*α while the qPCR was designed to measure closer to the 5' end of the mRNA in order to cross a predicted intron-exon boundary as is suggested for qPCR [9]. One possible explanation for the differences in the TSH cycles between the two techniques may be that that multiple transcripts were detected by one assay and not the other, but I have no evidence to suggest multiple transcripts for either gene product [15]. It is also possible that overexposure of the dot blots before quantification dampened differences between the time points.

Studies across vertebrate species have indicated that the TSH receptor is not confined to the thyroid gland [4]. Vertebrates including humans, mice, and chicken express the TSHR in the pituitary localized to folliculo-stellate cells (cells present between endocrine pituitary cells) [7, 29, 68]. Grommen et al. [29] proposed that the function of the TSHR on these folliculo-stellate cells was conserved between birds and mammals, and linked the pituitary TSHR expression to paracrine negative feedback within the pituitary. In this model the folliculo-stellate cells bind TSH which activates Dio2 conversion of T_4 to T_3 to promote short loop negative feedback. I similarly found the *TSHR* expressed in the red drum pituitary. Expression of *TSHR* in the first cycle experiment peaked as T_4 levels peaked which would be expected if the TSHR in the red drum does indeed play a role in intra-pituitary negative feedback. Unfortunately sufficient

RNA was not obtained in the second cycle experiment to further explore the cyclicity of the *TSHR* in the red drum pituitary. As noted in the introduction, TSH and the TSHR are expressed in the gut and regulate the activity of intestinal lymphocytes [88]. I found, in red drum, the *TSHR* and both TSH subunits expressed together in the gut suggesting that this intestinal signaling pathway might be a primitive function of TSH.

The expression of *TSHβ, GSUα, Dio2, Dio3*, and *TSHR* were detected in both laboratory-reared and wild red drum pituitaries. The higher expression of *TSHβ* in captive fish compared to wild fish also has been observed in other fish species but the significance of this difference is speculative given it has only been observed in the rainbow trout, Atlantic salmon, and now red drum [36, 58] and has not been subjected to careful physiological scrutiny. Because the GSUα subunit is coupled with TSHβ or the gonadotropins, measurements of GSUα are complicated by influences from the reproductive state of the fish. To avoid this complication, it has been suggested that juvenile, as opposed to adult fish, would be more useful for studying the regulation of the alpha subunit [15]. In my study, as predicted, I observed that *GSUα* correlated far more closely with *TSHβ* in juvenile than adult red drum supporting the assertion that expression of the alpha subunit in juvenile fish is largely tied with thyroid activity. Variations in TSHR have been linked in mammals with a TSH signaling pathway from the pituitary to the hypothalamus to regulate seasonal breeding [31, 32]. The higher

expression of pituitary *TSHR* seen in adult red drum coupled with the changing pituitary expression between the months sampled suggests a role for pituitary *TSHR* expression in red drum seasonality.

Of all fish studied, now including the red drum, *TSHR* expression has been discovered in the gonads of every species, save the amago salmon, suggesting that paracrine TSH signaling may play a role in gamete development [55]. TSH expression has been observed in the fathead minnow gonad [50], and also in the grouper where *TSH*β expression was found to be higher in the male gametes than the female gametes [89]. The expression of the *TSHR* in the testis was higher than the ovary possibly due to the large number of gametes in the testis. In red drum, gametes begin developing in July and August ahead of fall spawning [16]. Gonadal co-expression of TSH subunits with increasing *TSHR* in red drum supports a paracrine role for TSH in the gonad associated with elevating gametogenesis as spawning nears. A study all from the same year would be needed to truly clarify the expression of these thyroid-related transcripts in wild red drum.

The significant changes in steady-state mRNA expression presented in this study are consistent with the magnitude of changes of significance in other fish studies such as those in the bighead carp [13]. Although I did not measure associated deiodinase activity in pituitary tissue, there is reason to believe that mRNA expression can be used as a reasonable index of enzyme activity. In tilapia,

experimentally induced hypothyroidism resulted in changes in hepatic deiodinase 2 and 3 activities paralleled by changes in the expression of their respective mRNAs, leading VanDerGeyten et al. [85] to conclude that regulation of hepatic deiodinase activity was primarily pretranslational. In trout, 7 day T_3 treatment decreased hepatic *Dio2* mRNA expression and T_4 outer-ring deiodination (presumably through decreased Dio2 activity) while hepatic *Dio3* mRNA expression and T_3 inner-ring deiodination (presumably through Dio3 activity) increased, leading Bres et al. [6] to concluded that the change in mRNA expression over time can provide a useful index of deiodination activity. Additionally, hypothyroid rats treated with T_3 show a steady decline over 24 hours in cerebral cortex Dio2 mRNA and activity, lending support to the suggestion that in the fish liver and mammalian nervous tissue at least deiodinase mRNA expression can be used to estimate changes in deiodinase activity [8]. In conclusion, I have shown that steady-state *TSH*β and *GSU*α mRNA is expressed reciprocally to circulating T_4 as would be expected if T_4 negative feedback is a significant mechanism in regulating the T_4 cycle in red drum. The expression of pituitary *Dio3*, with the same pattern as the TSH subunits also reciprocal to T_4 levels, suggests Dio3 has an important role in the regulation of TSH expression by controlling intracellular availability of T_3 for negative feedback. T_3 must be produced for negative feedback but T_4, not peripherally produced T_3, cycles dynamically in red drum. Therefore the issue of feedback through T_3, the active hormone, versus T_4, the principal output from

the central mechanism at the thyroid gland, requires further examination. Given the variations of pituitary *TSHR* expression in the red drum, this transcript may also play a role in feedback and/or seasonality. Co-expression of TSH and the *TSHR* in the gonads and intestine suggest paracrine roles for TSH in these tissues. Overall, these experiments show that *TSHβ, GSUα, Dio2, Dio3*, and T*SHR* are robustly expressed in patterns consistent with an important contribution of pituitary thyrotrophs in central regulation of thyroid function in the red drum.

CHAPTER III

NEGATIVE FEEDBACK REGULATION OF *TSHβ, GSUα, DIO2, DIO3*, AND *TSHR* IN RED DRUM, *SCIAENOPS OCELLATUS*

Introduction

All vertebrates produce thyroid hormones which regulate growth, development, reproduction, metamorphosis, and metabolism [17, 21, 22, 84, 91]. Thyroid function in mammals is predominantly regulated by a central pathway through which hypothalamic neurohormones control the production of the GSUα/TSHβ heterodimer thyrotropin (TSH) in the pituitary gland. The TSH heterodimer binds to the TSH receptor (TSHR) in thyroid epithelial cells to stimulate the release of thyroid hormones (TH) from the thyroid gland. The principal thyroid hormone is the prohormone 3,5,3',5'-tetraiodothyronine (T_4) [83]. In target cells T_4 can be inactivated by an inner-ring deiodinase (IRD, Dio3) to form TH metabolites or activated by an outer-ring deiodinase (ORD, Dio2) to form 3,5,3'-triiodothyronine (T_3) which binds to nuclear receptors to elicit a TH response [21]. Both THs are known to negatively regulate TSH production in mammals [11].

In lower vertebrates, regulation of thyroid function is less well characterized. Lack of a known TSH molecule in agnathans suggests that vertebrate ancestors spontaneously released T_4 into the circulation and primarily

regulated thyroid hormone stimulation of target tissues through modulation of deiodinase activity in peripheral tissues, independent of central mechanisms [21]. Early studies in fish, primarily from salmonid species, supported a hypothesis that in teleosts thyroid hormone delivery to target cell receptors was predominately regulated by peripheral cellular mechanisms. Eales and Brown [7] summarized this evidence: a lack of circulating T_3 response to T_4 administration, a growing list of physiological variables that directly regulate peripheral deiodinases, and an apparent lack of negative feedback regulation of TSH production by blood T_3. They subsequently suggested that TSH's role in driving TH utilization may not be as significant in fish as it is in higher vertebrates.

Recent evidence in fish of a robust daily rhythm of circulating T_4, hypothesized to be driven by a central oscillator working through TSH in red drum, suggests that TSH may indeed be dynamically regulated by central mechanisms and play a more important role in the activation of fish thyroid function than previously expected [48, 49]. I have shown that the T_4 cycle in red drum is inversely related to the pituitary expression of mRNA for TSH, suggesting that this cycle is regulated by negative feedback of T_4 acting upon TSH (Chapter 2). *Dio2* was expressed in the pituitary at the peak of the T_4 cycle, but *Dio2* expression did not predictably and consistently vary (Chapter 2). In red drum, the role of Dio2 in feedback is thus still unclear. *Dio3* expression, on the other hand, was robustly cyclic with a waveform inversely related to circulating T_4, suggesting that TH negative feedback may be mediated in part through

regulation of intra-pituitary deactivation of THs (Chapter 2). I also showed that the *TSHR*, possibly functioning through an intra-pituitary negative feedback pathway [29], was expressed in the pituitary at the peak of the T_4 cycle suggesting a role for this protein in feedback as well (Chapter 2).

The relative importance of T_3 versus T_4 in negative feedback regulation of TSH secretion in fish is an important consideration in establishing the role of central mechanisms in regulating fish thyroid function [55]. In mammals, circulating T_4 drives T_3 utilization in peripheral cells and feedback regulation of TSH production at the pituitary. Negative feedback directly from circulating T_3, and of T_3 produced from circulating T_4 through deiodination within the pituitary gland are key components of the classical, mammalian model for central regulation of thyroid function [21]. The lack of evidence for T_3 negative feedback in fish was a major argument used by Eales and Brown [21] to support their contention that fish regulate thyroid function at the tissue level through a peripherally generated T_3 set-point instead of through a T_4 set-point regulated at the pituitary. The concept of a regulated T_3 set point in fish is consistent with the observation in many fish species that circulating T_3 circulates at much higher levels than in tetrapod vertebrates [12], suggesting that peripherally-deiodinated, recirculated T_3 may also serve an important role in achieving thyroid hormone stimulation of targets. Consistent with mammalian studies, T_4 or T_3 negative feedback control of TSH has been observed in several nonmammalian species [12, 28, 55, 57] but has been rarely studied in fish.

The few studies which have examined feedback regulation of TSH in fish have generally used pharmacological treatments. Inhibiting thyroid hormone synthesis by administration of goitrogens consistently elevates TSH expression in fish [55], providing evidence that negative feedback is important for the regulation of fish thyroid function. Goitrogens, however, often elicit toxic side effects. *In vivo* studies with fish such as the coho salmon, goldfish, or turbot have also demonstrated T_4 or T_3 suppression of TSH expression [55] or T_3 suppression of circulating T_4 [43, 66], but these studies used either pharmacological doses or durations of administration lasting 2 weeks or more, leaving the significance of circulating thyroid hormones in the physiological regulation of negative feedback in fish unresolved. Additionally, i*n vivo* experiments have methodological limitations. They have either utilized T_3 feeding exclusively, because T_4 is not absorbed from the diet [43, 50, 66], prolonged (weeks) of immersion in thyroid-hormone containing water, implantation of thyroid hormone pellets, [56, 67, 94], or injection of THs [15] using potentially stressful protocols [18]. Recently, a pituitary T_3 negative feedback mechanism that chronically suppressed TSH expression in a dose dependent manner at physiological concentrations *in vitro* was observed in the European eel [74]. A similar pattern of T_4 negative feedback has been observed *in vitro* in the bighead carp [13]. However, the important issue of establishing a differential sensitivity of central regulation to rapid changes in T_4 and T_3 *in vivo* has not been addressed. Leiner et al. [49] employed a T_3 immersion technique

in red drum that, by elevating T_3 over a brief period of hours, provided evidence for negative feedback of physiological doses of T_3 by significantly depressing the daily peak in circulating T_4 in red drum. A similar experimental design could be utilized to noninvasively administer not only T_3 but also T_4 to red drum to address the question of their relative importance in negative feedback. Using this method, it should be possible to address the physiological significance of negative feedback on TSH through T_3 vs. T_4, an important question to answer in addressing the hypothesis that fish thyroid function is primarily driven by peripheral mechanisms.

The overall objective of this study was to determine if physiological doses of T_3 and T_4 can sensitively and rapidly (in hours, not days) inhibit the expression of TSH. Additionally, my results in Chapter 2 suggested that circulating T_4 may also regulate the expression of pituitary proteins in addition to TSH, including potentially the deiodinase enzymes and the TSH receptor (TSHR). The qPCR techniques I developed in Chapter 2 should, for the first time, provide an opportunity to simultaneously measure the effects of *in vivo* T_3 and T_4 administration on both thyroid hormone subunits and deiodinase expression in fish pituitary glands. Sensitive down regulation of pituitary TSH expression by both T_3 and T_4 in addition to TH regulation of pituitary deiodinase expression would support the importance of central mechanisms acting in fish. If central regulation is important, I hypothesize that in red drum the pituitary expression of *TSH*β and *GSU*α will be inhibited by both T_3 and T_4. I further

hypothesize that, because Dio2 activity plays an important role in providing T_3 for negative feedback (Chapter 2), *Dio2* expression should be activated by physiological levels of T_4 at the pituitary to provide T_3 for negative feedback. In contrast, recent studies in mammals and from Chapter 2 have suggested that Dio3, through its catabolic actions, may be important in releasing central mechanisms from feedback inhibition [2, 76]. I therefore further hypothesize *Dio3* expression will be inhibited by THs at the pituitary. Additionally, ectopic expression of TSHR in the pituitary has been proposed to play a role in negative feedback on TSH by binding TSH and activating local T_3 production [29]. Data from Chapter 2 suggested that pituitary *TSHR* in red drum might be up regulated by TH for negative feedback. I therefore hypothesize that pituitary *TSHR* expression will be stimulated by TH administration in red drum. Finally, these *in vivo* studies have also provided the opportunity to assess the effects of thyroid hormone administration on ectopic expression of thyroid-related genes in the intestine. I have found previously (Chapter 2) that *TSHβ, GSUα,* and *TSHR* are co-expressed in the red drum intestine. Given that locally produced TSH (regulated by locally produced TRH) may serve to regulate immune function in the mouse intestine [88], the regulation of these transcripts in the red drum intestine warrants further research. Therefore, specific objectives of this study are: to develop a non-invasive technique for administering physiological doses of T_3 and T_4 that results in rapid reversible, physiological increases in blood hormone levels; to characterize differential negative feedback capabilities of T_3

vs. T_4 by measuring the pituitary mRNA expression of *TSHβ, GSUα, Dio2, Dio3*, and *TSHR* during constant TH immersion or goitrogen administration; and to gather evidence that intestinal expression of TSH subunits or the *TSHR* are regulated in a similar fashion by circulating thyroid hormones. This study will thus address the issue of how negative feedback through T_3 and T_4 might be achieved in red drum, thus providing additional information on the potential contribution of central mechanisms to the regulation of fish thyroid function.

Materials and methods

Animals

The CCA/CPL Marine Development Center in Corpus Christi or the Texas Parks and Wildlife Department's hatchery at Sea-Center Texas in Lake Jackson provided red drum fingerlings weighing approximately 0.5 g. Fingerlings were then held in the Department of Biology's BioAquatics Facility at Texas A&M University until they reached a weight of at least 20g before being used for experiments. Animals were housed in a 4000L recirculating system at 4ppt salinity (SuperSalt, Fritz Industries, Mesquite, Texas), 26C, 12L:12D photoperiod. Fish were fed Aquamax (PMI Nutrition, Brentwood, MO) to apparent satiation once daily. TH immersion experiments were performed in 80L tanks connected to the 4000L recirculating system. Fish were euthanized in MS-222 (Finquel, Argent Laboratories, Redmond, Washington) prior to obtaining

blood, pituitaries, liver, and intestine samples. All procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee.

Immersion Technique

The thyroid hormone immersion technique of Leiner and MacKenzie [49] was modified to develop my T_3 and T_4 immersion protocol. On the day before an experiment fish were moved to flow-through 80L tanks, all connected to the same recirculating system as their stock tank. At the beginning of the experiment flow to these tanks was turned off, their volume adjusted to 60L, and 3ml of either 0.1N NaOH vehicle or thyroid hormone solution (3,5,3',5'-tetraiodothyronine,T_4 or 3,5,3'-triiodothyronine,T_3, both from Sigma Chemical Co., St. Louis), diluted in 0.1N NaOH was added to control or treatment tanks, respectively. The T_3 dose was 3ml of a 2mg/ml T_3 solution (final target concentration of 100ng/ml tank water). For experiments 1 and 2 the T_4 dose was 3ml of a 2mg/ml T_4 solution (final target concentration of 100ng/ml tank water), but the T_4 dose in subsequent experiments was increased to 3ml of a 4mg/ml solution (final concentration 200ng/ml tank water). In experiments 1, 2, and 3, while the doses of T_3 and T_4 were being established, the immersion lasted for 4h before flow was returned to the tanks, but in experiments 4, 5, and 6, this time was extended to as long as 66h to examine the time course of blood and tissue response to TH immersion. At the conclusion of each experiment as much water as possible (approximately 90%) was removed from TH treated tanks, discarded

from the recirculating system, and replaced with hormone-free system water. Remaining water draining from these tanks back into the recirculating system was passed through activated carbon to remove any residual immersion hormone from the system.

TH Immersion Experiments

For experiments 1, 2, and 3, groups of five to seven fish per tank (mean weight 48.95±1.98g, 60.5±2.98g, and 19.65±.55g respectively) were sampled. Experiments 4, 5, and 6 were immersion experiments lasting either 4.5h, 22 and 40h, or 66h, respectively. Data from these three experiments were graphed together to facilitate visual comparison of changes in hormone levels and gene expression over a 66h period of immersion. Five to seven fish per group of 22.52±0.80g, 24.38±1.02g, and 29.93±1.97g were sampled for the 4.5h, the 22 and 40h, and the 66h experiments, respectively. To evaluate stability of hormone levels in water over time, water samples were taken from one tank for each treatment at regular intervals (see Results) throughout the 66h immersion in Experiment 6.

Goitrogen Experiment

This experiment has been previously described in Cohn et al. [15]. I reanalyzed pituitary mRNA from this experiment using qPCR, measuring the samples for previously unanalyzed *Dio2, Dio3*, and *TSHR* mRNA expression. Briefly, once daily ip injection of control saline or 100ug/g body mass

methimazole (MMI, Sigma), made fresh daily, was administered for 12 days to 240 fish (135±3.1g). Approximately 40 pituitaries were pooled for each replicate yielding three pooled samples for each treatment group.

Intestinal TSH expression: feeding and immersion experiments

A pair of pilot experiments were undertaken to determine whether thyroid hormone immersion and feeding influenced intestinal TSH and deiodinase expression. The thyroid hormone immersion technique used was based on Leiner and MacKenzie [49] and described above. On the day before the immersion experiment 5-6 fish (132.57±13.74g average weight) were moved from the stock tank to 80L tanks connected to the same recirculating system. On the initial day of this experiment flow to these tanks was turned off, the volume adjusted to 60L, and 3ml of either deionized water vehicle (control) or T_3 (sodium salt) 2mg/ml in deionized water (final concentration 100ng/ml tank water). An additional group was immersed in thyrotropin releasing hormone (TRH, Sigma) 15mg/ml in deionized water (final concentration 750ng/ml tank water). The T_3 was administered to tank water just once but the TRH dosing was repeated every 24h. Water was not changed or circulated during the 66h immersion period. Biological and mechanical filtration was provided by a previously conditioned corner filter in each tank. The fish were fed once daily in the morning. After 66h fish were terminally anesthetized, blood was sampled from the caudal vasculature, and fish were then dissected for intestine samples.

The objective of the feeding experiment was to determine whether hormone could be effective if applied (in the food) directly to the luminal side of the intestinal epithelium. Diet for the feeding experiment was prepared using the protocol of Larsen et al. [43] by spray application to the Aquamax (PMI Nutrition) food of either 0.02N NaOH EtOH vehicle (control), T_3 0.05mg/g food, or TRH 0.6mg/g food. For this feeding experiment, fish (96.51±5g average weight), acclimated to stock tanks for several weeks, were fed 10g of the experimental diets shortly before lights out the first day of the experiment and then again shortly after lights on the next morning. Eight hours after the second feeding, fish were terminally anesthetized, blood sampled from the caudal vasculature, and then dissected for intestine samples. Intestines were sampled from 5-6 fish per group that had obvious food present in the gut at the time of dissection. All samples were analyzed for TSH expression while, due to sample and supply availability, intestinal deiodinase expression was only measured from samples in which T_3 was applied (in the food) directly to the luminal side of the intestine.

Blood and tissue analysis

Blood was collected from the caudal vasculature and plasma was separated and frozen at -80C until analyzed for THs using Coat-A-Count Total T_4 or T_3 kits (Siemens, Los Angeles, CA) following the manufacturer's protocol as described by Cohn et al. [15]. Following terminal anesthesia, tissues for RNA extraction were rapidly removed by dissection and transferred to either TRIzol

(Invitrogen, Carlsbad, CA) for livers, RNeasy Plus Mini kit reagent (Qiagen, Valencia, CA) for intestines, or to ZR RNA Microprep kit reagent (Zymoresearch, Irvine, CA) for pituitaries. The dorsal cranium and then the brain were removed during dissection to expose the pituitary. The liver and intestine were exposed during dissection by cutting along the ventral midline from the cloaca to the lower jaw. All samples were frozen at -80C until RNA extraction according to the manufacturers' protocols. GlycoBlue (Invitrogen) was added during the TRIzol extractions to visualize the pellets.

qPCR

qPCR primers and probes are listed in Chapter 2, table 2.1. All RNA samples were treated with DNAse I (Zymo Research) according to the manufacturer's protocol prior to PCR. qPCR step 1 RT reactions and step 2 PCR reactions were performed using the High-capacity cDNA Reverse Transcription Kit and TaqMan Universal PCR Master Mix (Invitrogen) with red drum specific qPCR primers/probes (Integrated DNA Technologies) following the manufacturer's protocol. qPCR primers/probes were designed using Primer Quest software from Integrated DNA Technologies and are listed in Chapter 2. Step 2 of the qPCR was performed and analyzed using the Applied Biosystems 7500 Fast Real-Time PCR machine following the manufacturer's protocol for relative quantification. As described by Bustin [9], expression of the 18S

ribosomal subunit was amplified as a housekeeping control gene for qPCR. The qPCR assays are identical to the assays previously described in Chapter 2.

Graphing and statistics

The ΔΔCt method was used to calculate qPCR results. Relative values correspond to the mRNA expression of the gene-of-interest/18S Ribosomal subunit. The average control relative value corresponds to the mean of all relative values from control animals within the same experiment independently determined for each gene-of-interest. mRNA expression graphs were displayed as percent of the average control relative value to eliminate any bias toward one experimental time point over another. Error bars represent standard error. SPSS software was used for Kruskal-Wallis followed by Mann-Whitney tests to determine significance at $p \leq 0.05$.

Results

Experiments 1, 2, and 3: Development of TH dose response to TH immersion

In experiment 1 (figure 3.1), both T_3 and T_4 were added simultaneously to individual tanks. This initial experiment was designed to determine whether T_4 immersion could elevate circulating T_4. As Leiner and MacKenzie [49] had already established that immersion in T_3 elevates red drum circulating T_3, T_3 was included as a positive control. In this experiment the magnitude of the resultant fish blood T_3 and T_4 elevations was approximately the same (T_3 was elevated to

11.78±0.91ng/ml and T_4 to 12.56±1.40ng/ml), and significant elevation of both hormones persisted in the blood until at least 4h after the flow was returned to the tanks. In experiment 2 (figure 3.2), the same dose of T_3 or T_4 alone was added to the water. In this experiment T_3 values in fish immersed in 100ng/ml T_3 were significantly higher than controls at both 2 and 16h after flow had been returned to the tanks (figure 3.2A). The T_3 response 2h after the flow had been returned (8.62±1.31ng/ml) was significantly higher than that of T_4 (5.22±1.06ng/ml), but in this experiment T_4 levels in animals immersed in 100ng/ml T_4 were not significantly different from controls (figure 3.2B). Immersion in 100ng/ml T_3 significantly reduced T_4 levels in these fish at both 2 and 16h after flow had been returned to the tanks (figures 3.2B). Measurements of tank water revealed that the hormones were no longer present in the water 30 minutes after the flow had been returned to the tanks (data not shown). In experiment 3 (figure 3.3), the dose of T_4 was doubled in an attempt to elevate circulating T_4 above control values (the dose of T_3 was not changed from previous experiments). In this experiment the maximum amplitude response of circulating T_3 (19.48±1.06ng/ml) 30 minutes after the flow had been restarted was not different from T_4 (19.02±1.00ng/ml) and both of these responses were significantly different from controls (figure 3.3).

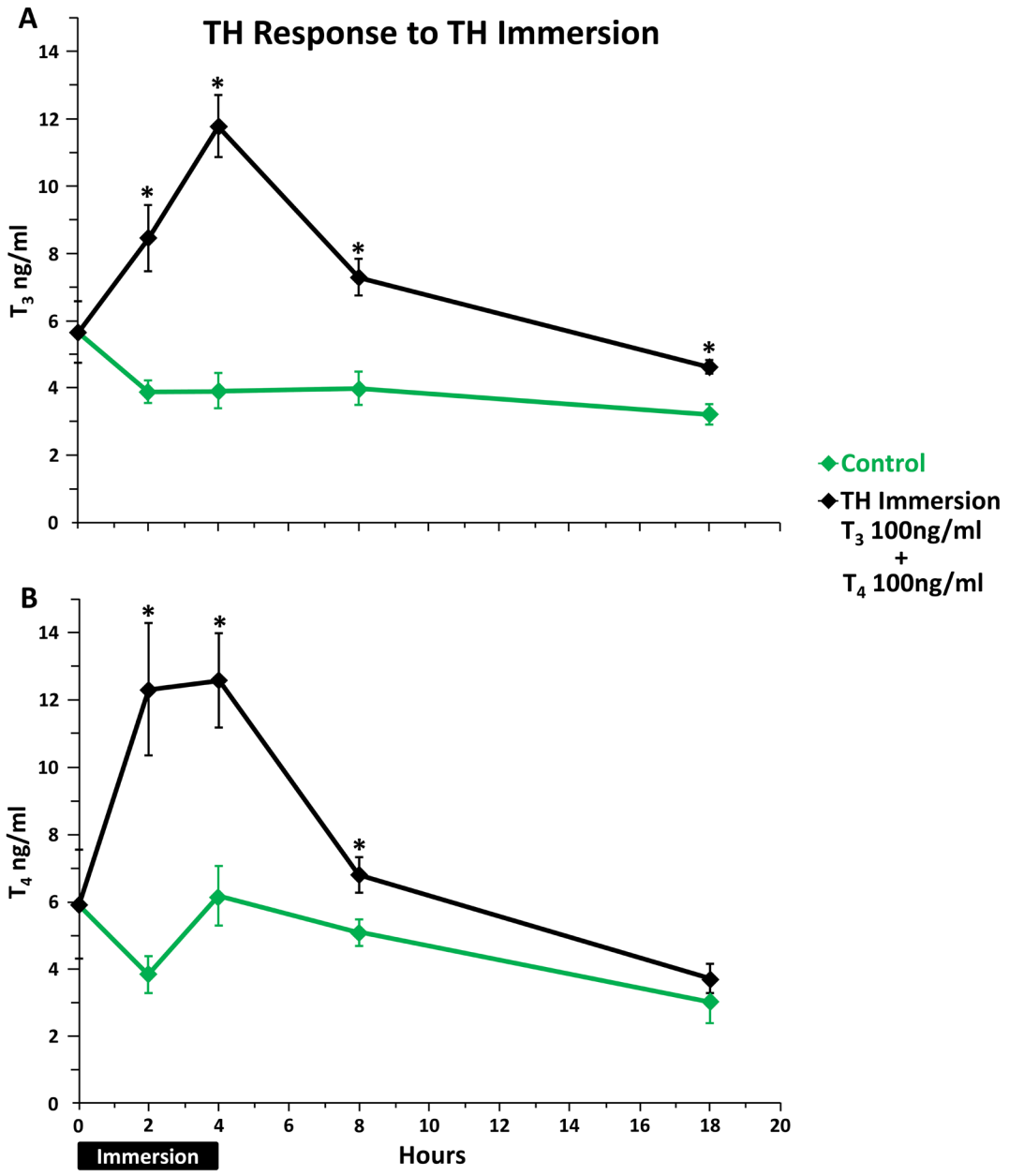

Figure 3.1. Ch3 Experiment 1: (A) T_3 and (B) T_4 response in red drum to 4h thyroid hormone immersion. Blood samples were taken from fish initially immersed for 4h in both T_3 (100ng/ml) and T_4 (100ng/ml) or NaOH vehicle before flow was returned to the tanks. Significant differences ($p \leq 0.05$) from control are denoted by asterisks. Sample size was 5-7 per time point.

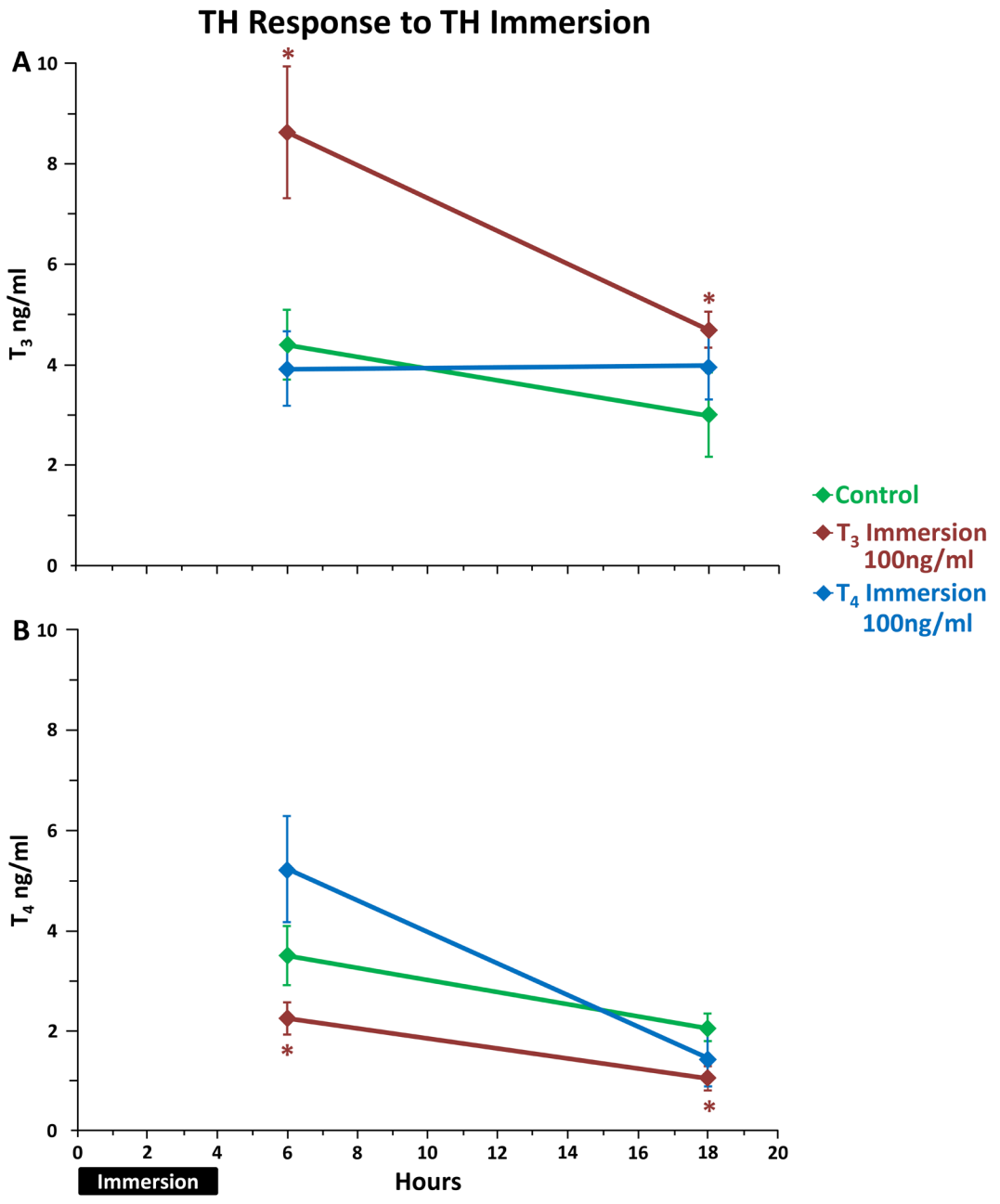

Figure 3.2. Ch3 Experiment 2: (A) T_3 and (B) T_4 response in red drum to 4h thyroid hormone immersion. Fish blood samples were taken beginning 2h after flow was returned to tanks initially immersed for 4h with either T_3 (100ng/ml) in red, T_4 (100ng/ml) in blue, or NaOH vehicle in green. Significant differences ($p \leq 0.05$) from control are denoted by asterisks. Sample size was 5-7 per time point.

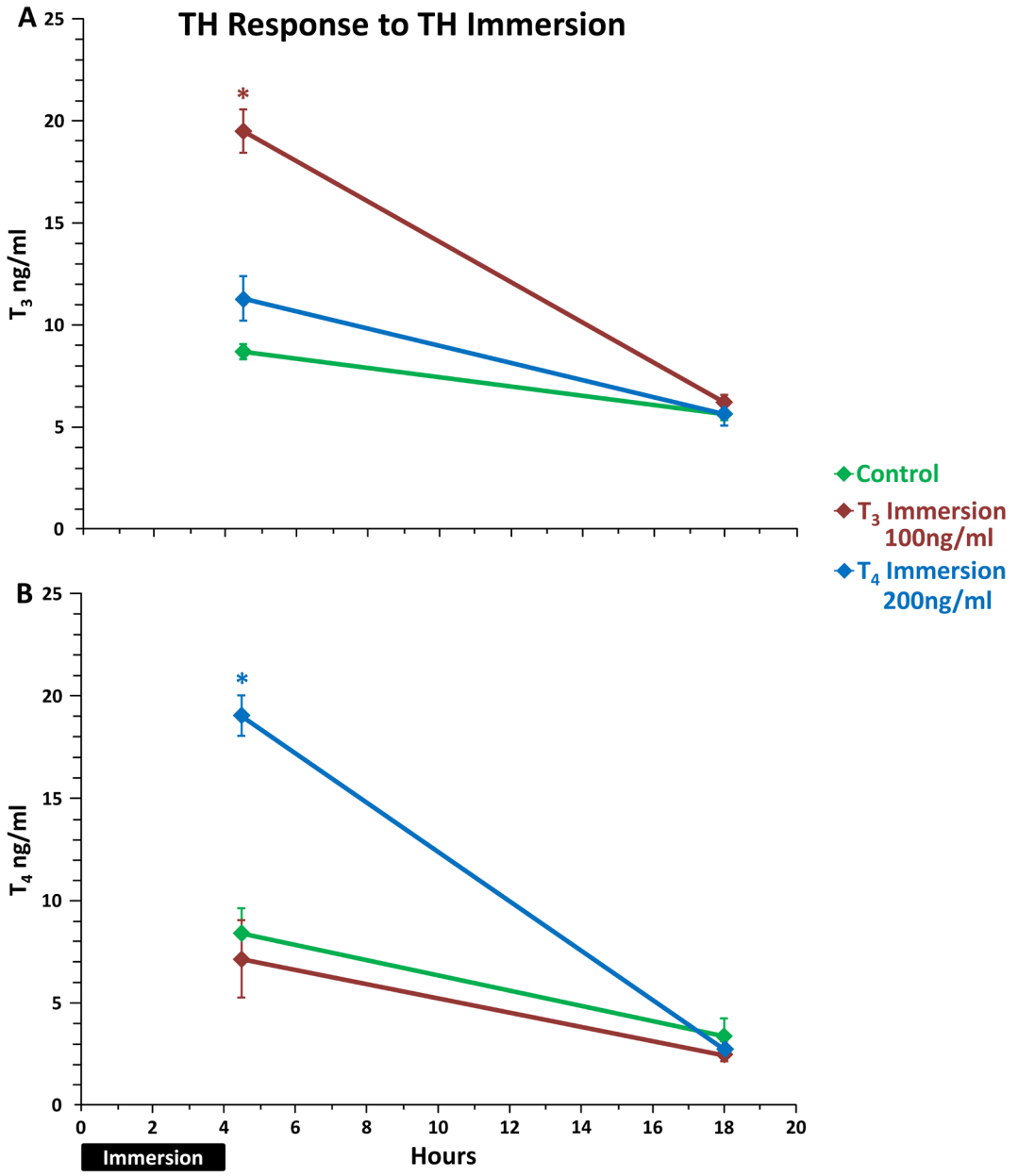

Figure 3.3. Ch3 Experiment 3: (A) T_3 and (B) T_4 response in red drum to 4h thyroid hormone immersion. Fish blood samples were taken beginning 30 minutes after flow was returned to tanks initially immersed for 4h with either T_3 (100ng/ml) in red, T_4 (200ng/ml) in blue, or NaOH vehicle in green. Significant differences ($p \leq 0.05$) from control are denoted by asterisks. Sample size was 5-7 per time point.

Time course of TH persistence in the water and blood of red drum

With the doses of T_3 and T_4 now established, the time course of the administration technique was examined. The length of immersions in the remainder of these studies was extended ultimately to 66h. The levels of T_4 in the T_4 immersed tank water (at 200ng/ml) were, as expected, higher than the levels of T_3 in the T_3 immersed tank water (at 100ng/ml) (figure 3.4), although both were below the target tank concentrations. Control tanks had minimally detectable (less than 0.2ng/ml) to undetectable THs in their water. The concentration of THs in tank water slowly decreased over time during the static immersion (figure 3.4). Blood from fish immersed in these T_3 or T_4 concentrations showed significant elevations of T_3 or T_4 between 4.5 and 40h, but only T_3 was elevated at the end of the 66h immersion (figure 3.5). T_4 immersion initially elevated blood T_3, but by 40h this response had reversed (figure 3.5A). T_3 immersion significantly depressed blood T_4 at every point other than the initial 4.5h sampling (figure 3.5B). Several efforts to immerse the fish in thyroid hormone synthesis inhibitors, such as methimazole (MMI), did not significantly alter blood TH levels (data not shown).

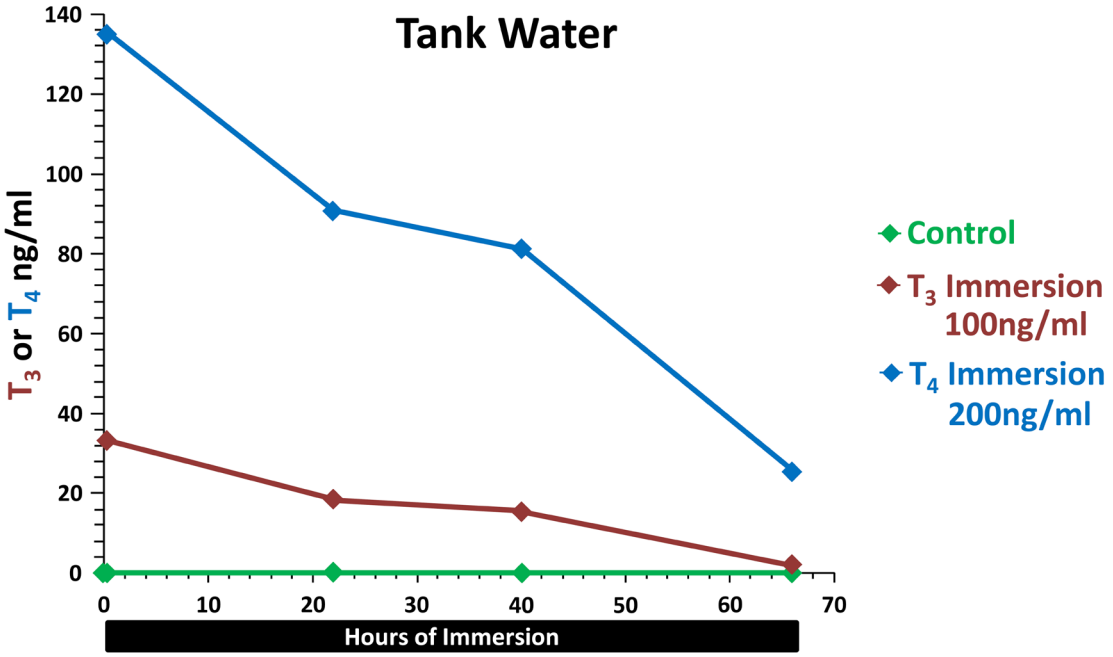

Figure 3.4. T_3 and T_4 in red drum tank water during 66h thyroid hormone immersion. Water was sampled from tanks treated with constant static immersion in 100ng T_3/ml water (in red), 200ng/ml T_4 (in blue), or NaOH vehicle control (in green) and measured in a T_3 or T_4 RIA.

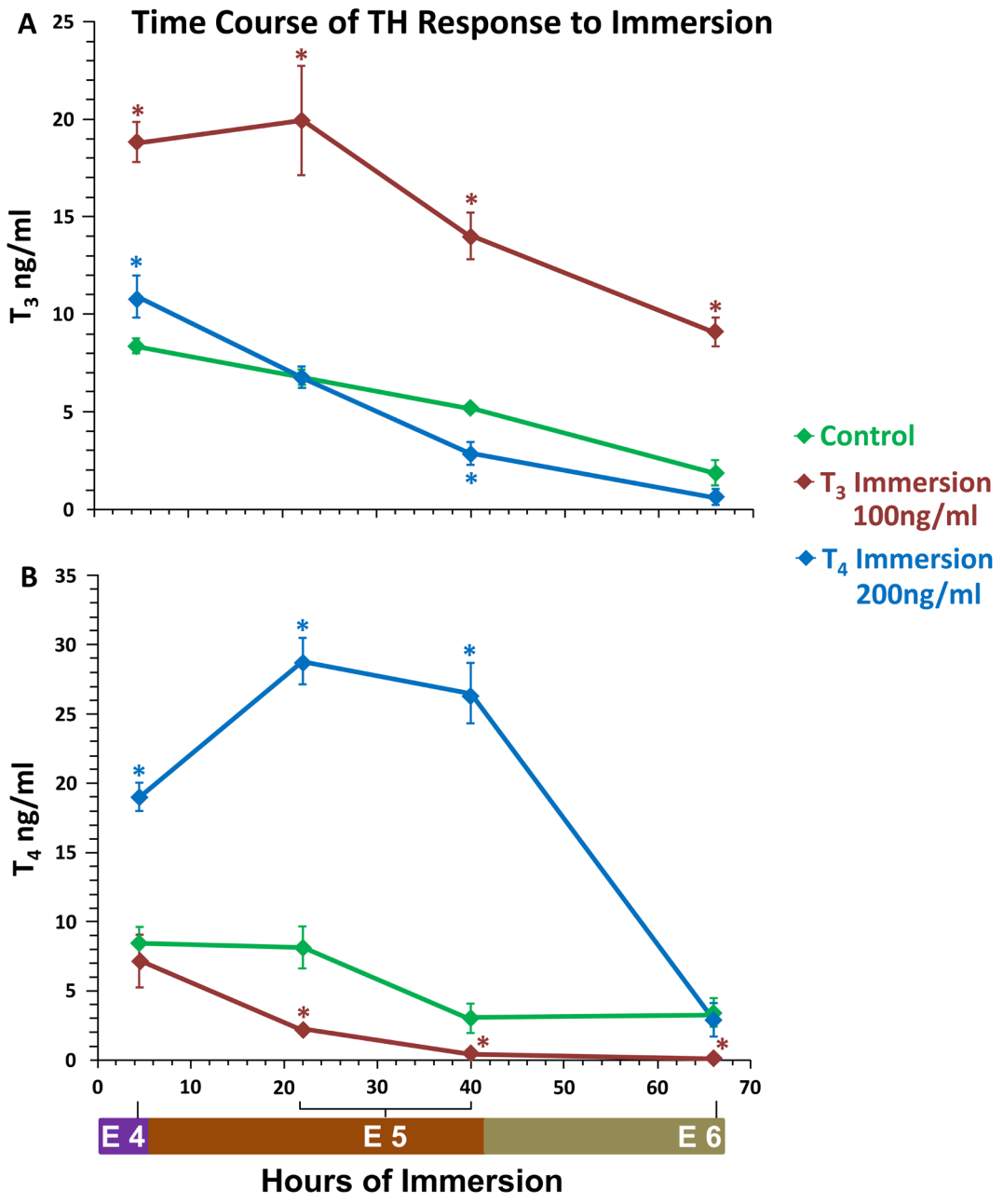

Figure 3.5. (A) T_3 and (B) T_4 response in red drum to up to 66h of thyroid hormone immersion. Blood samples were taken from fish in constant static immersion with either T_3 (100ng/ml) in red, T_4 (200ng/ml) in blue, or NaOH vehicle in green. Experiment 4 (E4), experiment 5 (E5), and experiment 6 (E6) are graphed together on a single axis. Significant differences ($p \leq 0.05$) from control are denoted by asterisks. Sample size was 5-7 per time point.

Effects of TH immersion on liver Dio2 and Dio3 mRNA expression

In the liver, at 22 and 40h, T_3 immersion significantly inhibited the expression of *Dio2* and stimulated the expression of *Dio3*. T_4 immersion did not alter the expression of *Dio2* or *Dio3* in the liver (figure 3.6).

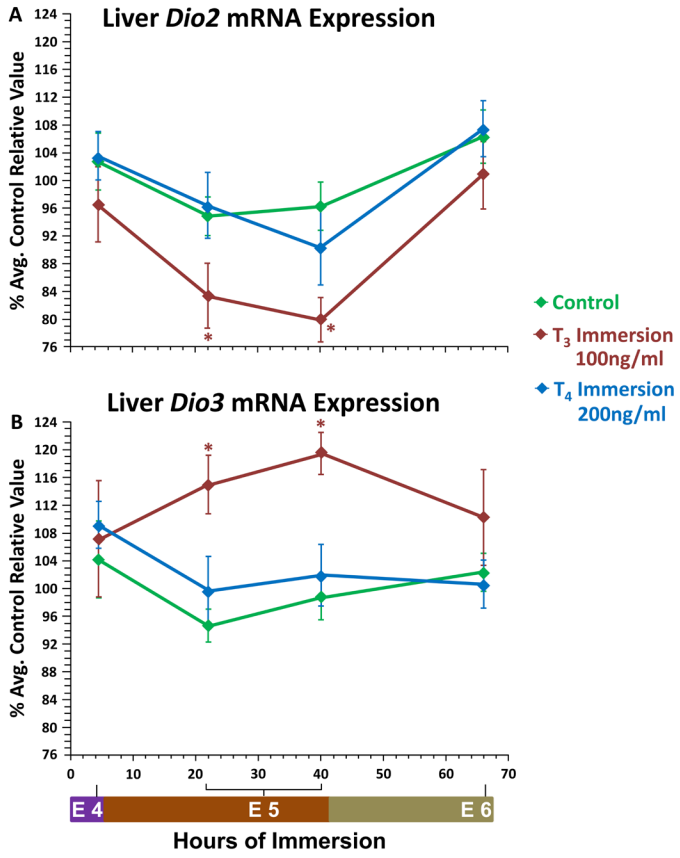

Figure 3.6. Red drum liver (A) *Dio2* and (B) *Dio3* mRNA expression resulting from thyroid hormone immersion. Liver samples were taken from fish in constant static immersion with either T_3 (100ng/ml) in red, T_4 (200ng/ml) in blue, or NaOH vehicle in green. qPCR was used to quantify (A) *Dio2* and (B) *Dio3* expression in liver RNA from T_3, T_4, and control immersed fish. Experiment 4 (E4), experiment 5 (E5), and experiment 6 (E6) are graphed together on a single axis. Significant differences ($p \leq 0.05$) from control are denoted by asterisks. Sample size was 5-7 per time point.

Effects of TH immersion on pituitary TSHβ, GSUα, Dio2, Dio3, and TSHR mRNA expression

Constant static immersion with T_3 or T_4 significantly inhibited the expression of *TSHβ* and *GSUα* at three out of four time points, with a maximal T_4 inhibition at 22h (figure 3.7). In contrast, *Dio2* expression was significantly different from control at only the 22h time point (figure 3.8A). T_3 and T_4 immersion significantly diminished the expression of pituitary *Dio3* with a maximal T_4 inhibition from control at 22h (figure 3.8B). *TSHR* expression was not significantly different from control at any time point during the TH immersions (figure 3.9). Pituitary samples from the MMI experiment performed by Cohn et al. [15] were re-analyzed, this time by qPCR, for *TSHβ* and *GSUα* expression. This same experiment was also analyzed for *Dio2, Dio3*, and *TSHR* mRNA expression (figure 3.10). Both THs were significantly depressed in blood samples from the MMI-treated fish. *TSHβ* and *GSUα* mRNA expression was significantly elevated while *Dio2* expression was significantly decreased in the MMI group (figure 3.10). MMI treatment did not alter the expression of *Dio3* or *TSHR* in the pituitary (figure 3.10).

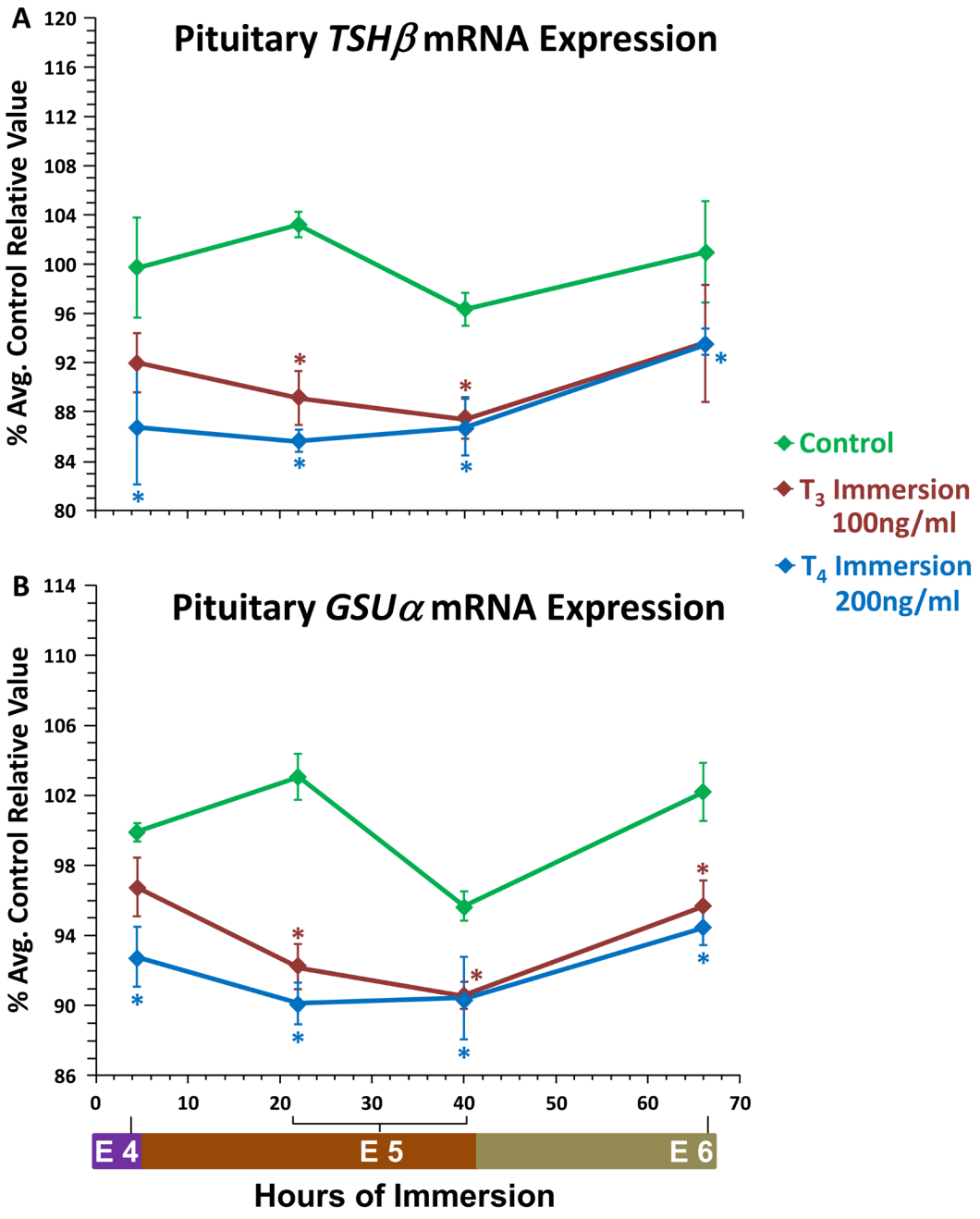

Figure 3.7. Red drum pituitary (A) *TSHβ* and (B) *GSUα* mRNA expression resulting from up to 66h of thyroid hormone immersion. Pituitary samples were taken from fish in constant static immersion with either T_3 (100ng/ml) in red, T_4 (200ng/ml) in blue, or NaOH vehicle in green. qPCR was used to quantify (A) *TSHβ* and (B) *GSUα* expression in liver RNA from T_3, T_4, and control immersed fish. Experiment 4 (E4), experiment 5 (E5), and experiment 6 (E6) are graphed together on a single axis. Significant differences ($p \leq 0.05$) from control are denoted by asterisks. Sample was 5-7 per time point.

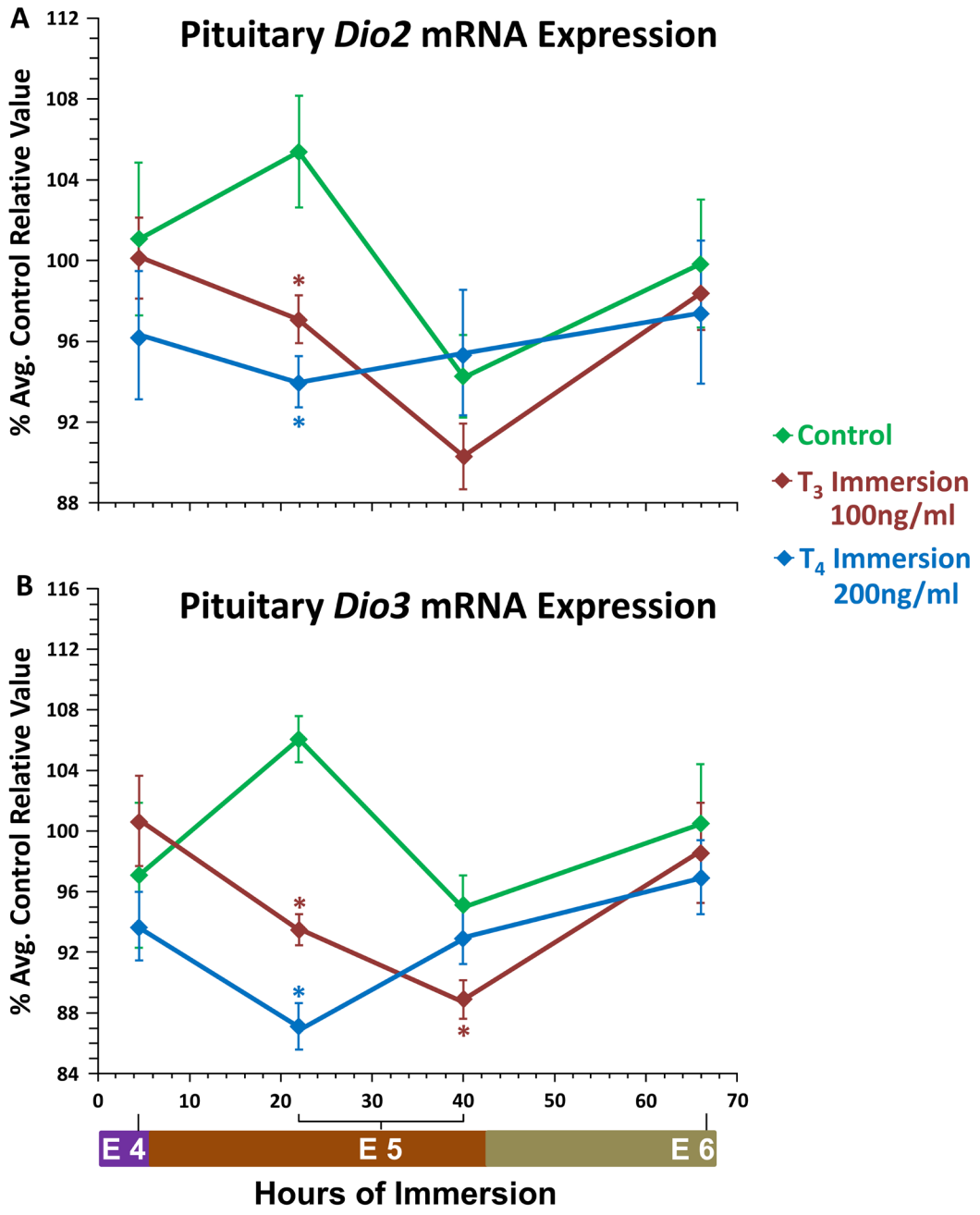

Figure 3.8. Red drum pituitary (A) *Dio2* and (B) *Dio3* mRNA expression resulting from up to 66h of thyroid hormone immersion. See figure caption 3.7 for axis details.

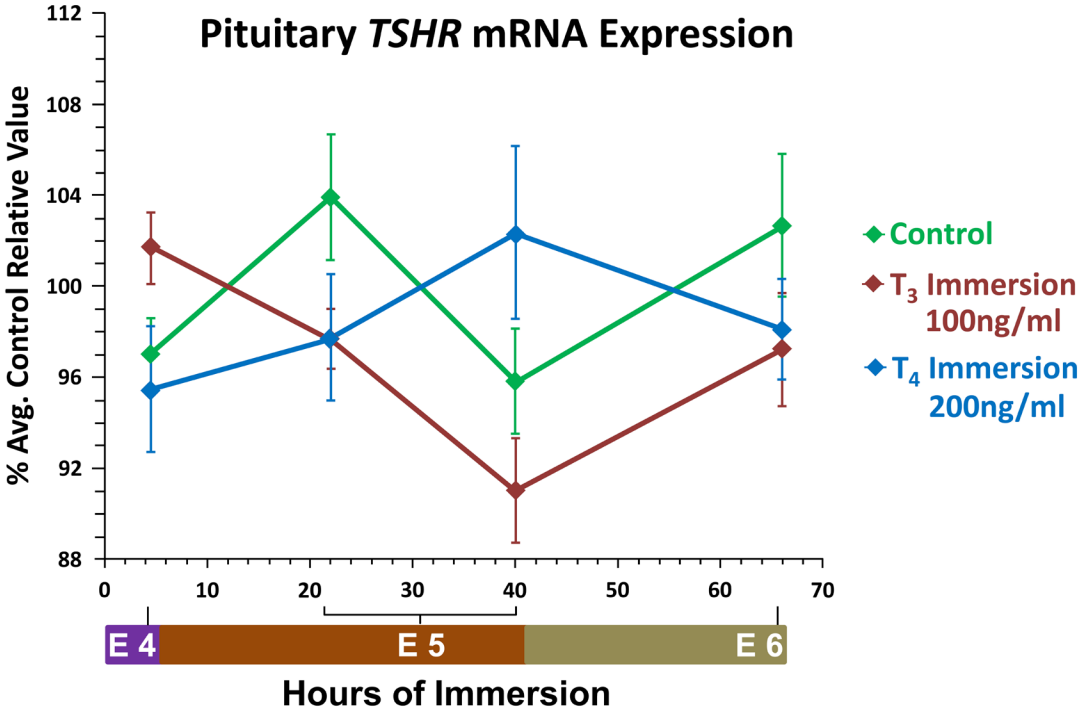

Figure 3.9. Red drum pituitary *TSHR* mRNA expression resulting from up to 66h of thyroid hormone immersion. Experiment 4 (E4), experiment 5 (E5), and experiment 6 (E6) are linked with dashed lines. See figure caption 3.7 for axis details.

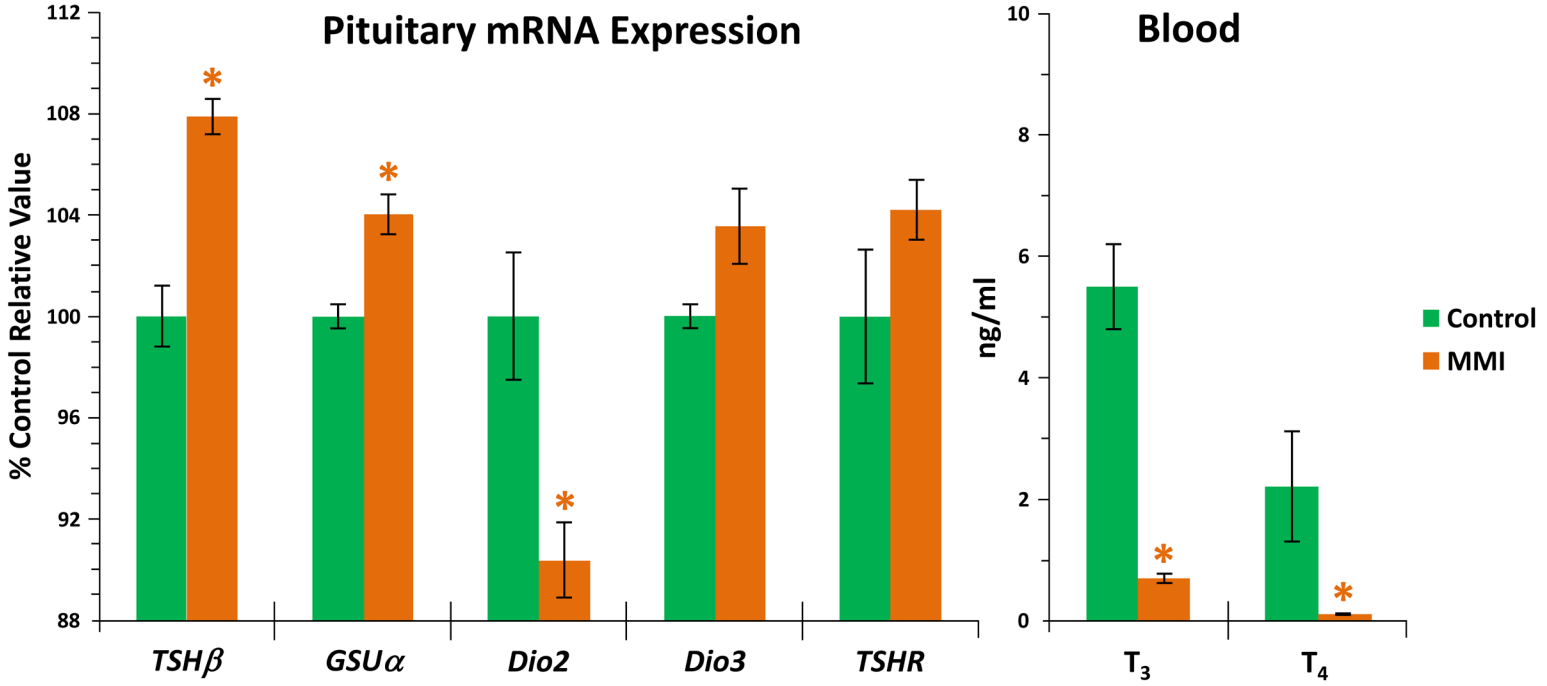

Figure 3.10. Pituitary *TSHβ, GSUα, Dio2, Dio3*, and *TSHR* mRNA expression in addition to blood T_3 and T_4 response in red drum to 12 days of methimazole (MMI) injections. Significant differences ($p \leq 0.05$) from control are denoted by asterisks. Sample size was 3 replicate pools per treatment or control.

Intestinal TSH and deiodinase mRNA expression in red drum

Only two significant changes in TSH subunit expression were observed in the intestine with immersion or hormone feeding (figure 3.11): Immersion with thyrotropin releasing hormone (TRH) significantly elevated *TSHβ* expression by 8.1% in the intestine whereas feeding T_3 significantly depressed *TSHβ* intestinal expression by 5.6% (figure 3.11). Feeding T_3 did not alter expression of *Dio2* in the intestine whereas the same treatment significantly increased *Dio3* intestinal expression by 22.6% (figure 3.12)

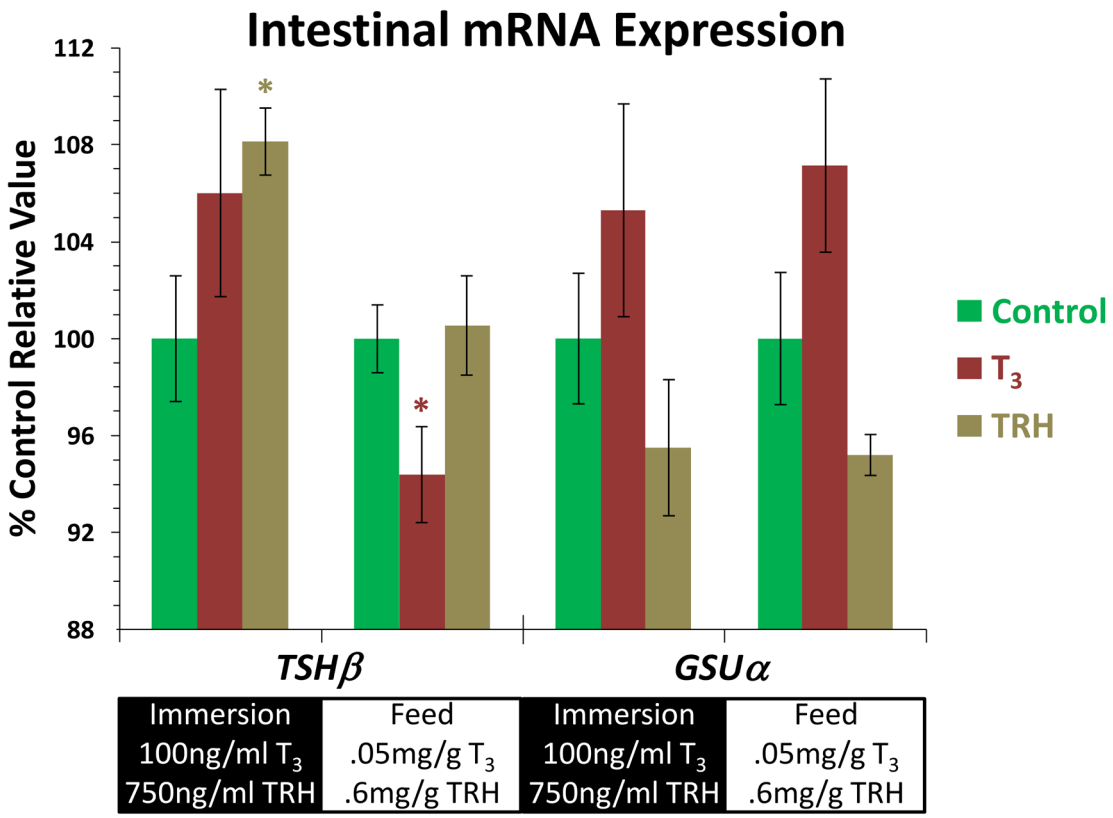

Figure 3.11. Regulation of intestinal *TSHβ* and *GSUα* mRNA expression in red drum. The expression of *TSHβ* and *GSUα* from the intestines of red drum treated with T_3 (red bars) or TRH (tan bars) are graphed in reference to control (in green bars). Expression is grouped by transcript (*TSHβ* or *GSUα*) and by application on the horizontal axis with immersion (in solid black) and feeding (in white). Significant differences ($p \leq 0.05$) are indicated by asterisks. Sample size was 5-6 per treatment or control.

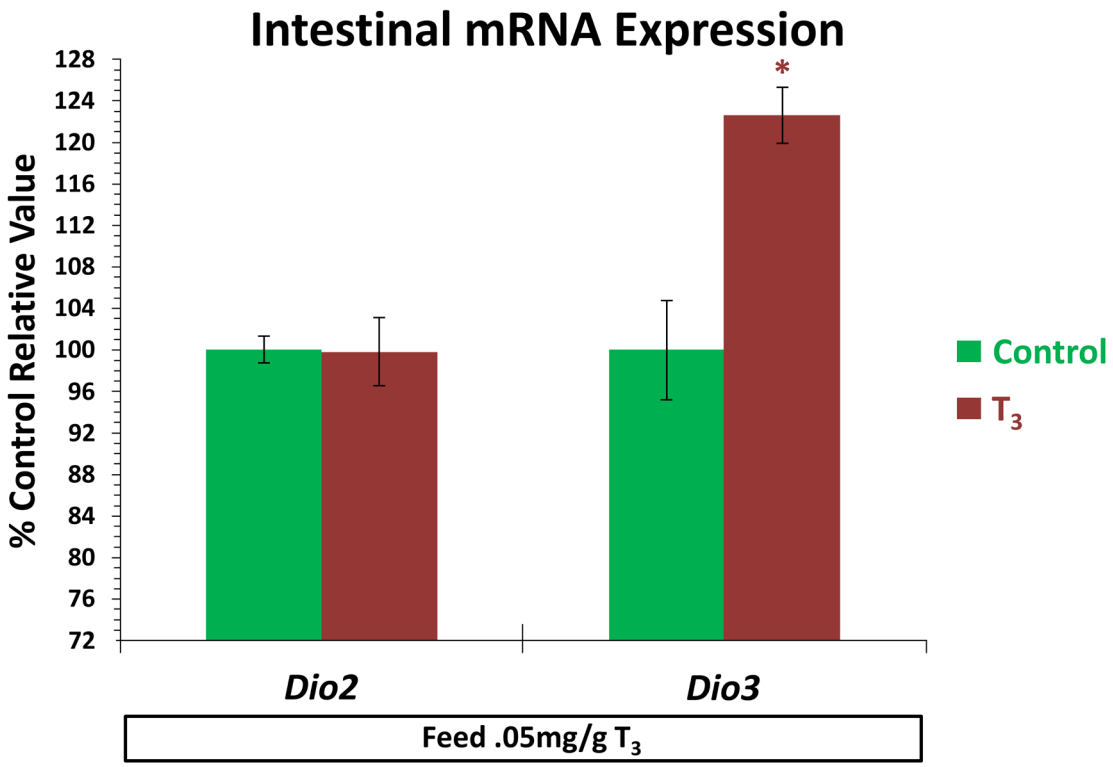

Figure 3.12. T_3 regulation of intestinal *Dio2* and *Dio3* mRNA expression in red drum. The expression of *Dio2* and *Dio3* from the intestines of red drum treated with T_3 (red bars) are graphed in reference to control (in green bars). Expression is grouped by transcript (*Dio2* or *Dio3*) both from T_3 feeding application on the horizontal axis. Significant differences ($p \leq 0.05$) are indicated by asterisks. Sample size was 5-6 per treatment or control.

Discussion

In this study I developed new techniques for noninvasively administering elevated doses of both T_3 and T_4 within a time frame replicating endogenous thyroid hormone cycles in the red drum. Using this technique I was able to measure physiological responses of circulating THs, liver deiodinase mRNA expression, pituitary *TSHβ, GSUα, Dio2, Dio3*, and *TSHR* mRNA expression to exogenous TH administration. Additionally, I measured the expression of pituitary *TSHβ, GSUα, Dio2, Dio3*, and *TSHR* mRNA expression during treatment with the goitrogen methimazole. The significant changes in steady-state mRNA expression presented in this study are consistent with the magnitude of changes of significance in other fish studies such as those in the bighead carp [13]. My studies suggested that both T_3 and T_4 were capable of eliciting negative feedback control on the synthesis of TSH. These techniques also enabled me to demonstrate expression of TSH in the intestine, a tissue known in mammals to express TSH.

A variety of techniques have been utilized to administer thyroid hormones to fish but most of these techniques, particularly injection, either lead to pharmacological elevations or induce stress and as such complicate interpretation of data [20]. Hormone feeding has been used extensively in rainbow trout, but this method fails to account for differential dosing due to variable food consumption among animals [20]. Additionally, feeding

experiments have traditionally focused on T_3 feeding because T_4 is not absorbed across the gut of fish [20, 43, 50, 66]. Immersion on the other hand is non-invasive and insures that all fish receive equivalent hormone exposure [20]. Leiner and MacKenzie [49] developed an immersion technique for administering T_3 which resulted in a down-stream reduction in circulating T_4, presumably through negative feedback at the pituitary in red drum, a species known for dynamic rhythms of circulating T_4 hypothesized to be driven centrally by TSH. This immersion protocol did not include T_4 as a treatment however. In order to examine the differential effects of T_3 vs. T_4 on feedback inhibition of TSH I therefore expanded this technique to T_4 administration.

Experiments 1, 2, and 3 were designed to establish the dose for the immersion technique. In experiment 1 T_3 and T_4 were added together to the water to confirm that fish were able to take up both hormones. Although this initial combined hormone experiment indicated that red drum were able to accumulate both hormones from their water, subsequent experiments indicated that when immersed in T_3 or T_4 alone, red drum took up T_3 more readily than T_4. In fact, the dose of T_4 used to achieve comparable levels of circulating T_4 in T_4 immersed fish was twice as high as the T_3 dose. Currently, the mechanism through which ambient thyroid hormone enters fish circulation is unknown. However, the monocarboxylate transporter MCT8, known to be a thyroid hormone transporter in mammals [23] is expressed in the zebrafish gill [1] and preferentially transports THs in a temperature-dependent fashion, with T_3

transport maximal at 26C vs. T_4 at 37C. If MCT8 in red drum is expressed in the gills and exhibits similar thyroid hormone transporting properties, at our water temperature of 25-29C, a preference of T_3 over T_4 uptake would be expected, as was observed. After effective physiological doses were established in experiment 3, responses to T_3 and T_4 immersion in red drum were characterized.

Experiments 4, 5, and 6 addressed the time course of responses to T_3 and T_4 immersion. Circulating T_4 significantly increased within 4h of initiation of immersion but eventually declined to control levels by 66h coinciding with the decline of T_4 in the water. Likewise, circulating T_3 also initially increased within 4h but its decline coincident with the decline in tank water content resulted in residual levels still above control by 66 hours. In my studies, T_4 was diminished in T_3 immersed fish by 22 hours presumably through T_3 negative feedback. T_4 was depressed to control levels in T_4 immersed fish by 66 hours even though a substantial concentration of T_4 was still present in the tank water. This depression in T_4 could also be due to feedback, in this situation via T_4 negative regulation of TSH expression. T_3 remained elevated in T_3 immersed fish even at 66 hours suggesting that while TH feedback regulates blood T_4, blood T_3 is instead regulated in part by a different mechanism. One such mechanism could act through deiodinase activity in the liver, as supported by previous studies [6] and by my data, demonstrating T_3 but not T_4 regulation of hepatic deiodinase expression. Intestinal bacteria degrade THs [19]. The gradual decrease in tank

water TH concentration over time could be due to spontaneous or bacterially-mediated hormone catabolism in the tanks, hormone degradation through prolonged exposure to light, or loss of hormone from the tank water in aerosols created by the tank's air stones.

Eales and Brown [21] cited a lack of T_3 feedback (as evidenced by no corresponding decrease in circulating T_4) and the inability of exogenous T_4 to raise circulating T_3 as evidence supporting the importance of peripheral regulation of thyroid function in fish. In mammals, well characterized for regulating thyroid function centrally, both T_3 and T_4 exert negative feedback at the pituitary and hepatic ORD functions to generate additional T_3 when circulating T_4 is elevated [21]. In fish, however, the hepatic regulation of deiodination has only been examined in a few species. Bres et al. [6] found that both *Dio2* and *Dio3* are expressed in the trout liver. Additionally, they observed that hepatic *Dio2* expression was down-regulated by T_3 whereas, liver *Dio3* expression was up-regulated by T_3. In their study neither *Dio2* nor *Dio3* expression were influenced by T_4. Hepatic regulation of a blood T_3 (not T_4) set-point is an important component of the peripheral regulation of thyroid function [21]. I also observed a similar differential hepatic response of *Dio2* and *Dio3* to T_3 vs. T_4 immersion, serving as confirmation that my *Dio2* and *Dio3* qPCR assays can detect expected physiological changes for these hepatic deiodinase gene products.

The observed changes in circulating T_3 in red drum immersed in T_4 has not been reported in fish before and are consistent with a temporal change in peripheral deiodination similar to what is seen in mammals. In this scenario, for the first 4h the elevated T_4 would enter target cells and be converted to T_3 by ORD to elicit peripheral TH target responses. Indeed, the bulk of circulating T_3 in fish is thought to be generated by T_4 deiodination [21]. T_3 returning to the circulation from these target cells would then be responsible for the early response of elevated blood T_3 observed at 4h. Since *Dio3* expression in the liver of fish has been found to be activated by T_3 and not T_4 (see figure 3.6 and [6]) the generation of this T_3 should progressively decline over time as hepatic expression of IRD increases, and circulating T_3 would be expected to fall. This delayed activation of IRD might thus explain the steady decrease of T_3 in T_4 treated fish, where by 40h circulating T_3 had significantly declined below control levels. The decreased circulating T_4 observed in T_3 immersed fish and those of Leiner and MacKenzie [49] is consistent with a negative feedback action of T_3 at the pituitary thyrotroph. Samples from T_3 immersed fish taken 6 (2h after flow was returned), 22, 40, and 66h into my experiment all showed a reduction in T_4 suggesting that T_3 has a sensitive and sustained negative feedback effect on TSH. My observations that T_4 can drive T_3 production and that T_3 can exert negative feedback at the pituitary both support an important role for central mechanisms in activating thyroid function in red drum.

Negative feedback of T_4 and T_3 on TSH expression at the pituitary gland is a key component of the mammalian central model for regulation of thyroid function [21]. In fish, *in vitro* inhibition of TSH expression by T_3 or T_4 has been observed, signifying that feedback of both hormones at the pituitary is physiologically possible [13, 74]. However, the *in vivo* physiological role of inhibitory thyroid hormone feedback on TSH expression in fish has not been consistently demonstrated [20]. *In vivo* experiments in fish have demonstrated T_4 or T_3 suppression of TSH expression, but usually employing pharmacological doses or prolonged time courses of administration of 2 weeks or more [55]. In red drum, I found that a dynamic daily rhythm of circulating T_4 is mirrored by cyclic expression of TSH (Chapter 2) suggesting that this TSH cycle is regulated by negative feedback at the pituitary. The red drum TSH cycle was 6-12h out of phase with circulating T_4 further suggesting that this negative feedback was occurring over a time scale of hours (Chapter 2). Thyroid hormone administration results in the present chapter provide additional support for this negative feedback model. Within 4h of the initiation of immersion T_4 had significantly inhibited the expression of both TSH subunits, indicating that T_4 within the range of circulating levels encountered during the daily cycle can feed back rapidly enough to inhibit TSH production and thus drive the TSH cycle in red drum. This negative feedback could be sustained for up to 66h, providing convincing evidence for a powerful negative feedback action of both of these hormones.

In mammals, T_3 for negative feedback on TSH expression is generated by Dio2 deiodination of T_4 [3, 63] thus making Dio2 critical for TH actions in the pituitary. Even though T_4 down-regulates *Dio2* expression in the mouse pituitary, sufficient T_3 is still produced for feedback [14]. In fish, ORD and IRD are known to be regulated by T_3 in the fish liver, but the TH regulation of deiodinase expression in the fish pituitary has, until now, not been studied. In the red drum I found that both T_3 and T_4 down-regulated the expression of *Dio2*, but given the situation in mice, this reduction in enzyme expression may not necessarily result in levels of intra-pituitary T_3 production insufficient for feedback. Conversely, in the mouse pituitary *Dio3* is up-regulated in the face of high circulating THs, likely preventing excessive inhibition of TSH secretion by degrading T_3 [2]. Interestingly, in the red drum pituitary I found the opposite result, where physiological levels of TH significantly inhibited the expression of *Dio3*. This also differs from the observed effect of T_3 on *Dio3* expression in the red drum liver.

Observations in tilapia that T_3 differentially regulates Dio3 in the brain, liver, and gill and in mice that *Dio2* is down-regulated by T_3 in the brain while up-regulated by T_3 in brown adipose tissue led Orozco and Valverde-R [65] and Johnson and Lema [37] to suggest that THs regulate deiodinase expression in a tissue-specific manner. Although no TH response element has been discovered in the human *Dio3* gene promoter, T_3 applied to GH3 cells cotransfected with a human *Dio3* gene promoter construct and the thyroid hormone receptor (TRα but not TRβ) stimulated luciferase activity from this *Dio3* promoter construct,

leading Barca-Mayo et al. [2] to suggest that differential expression of the TRs may mediate tissue-specific stimulatory vs. inhibitory regulation of Dio3 in response to TH. Alternatively, T_3 has been suggested to negatively regulate TSH expression in the pituitary through direct activation of co-repressors [11, 79]. A similar mechanism of co-activator or co-repressor direct regulation could be involved in TH mediated expression of deiodinases allowing for inhibition of expression in tissues such as the pituitary and stimulation in tissues such as the liver. No information is currently available on the molecular mechanisms through which thyroid hormones up- or down-regulate gene expression in fish tissues.

In Chapter 2 I found in red drum that cyclic expression of TSH was temporally matched with pituitary *Dio3* expression and mirrored by a dynamic daily rhythm of circulating T_4, suggesting that the TSH subunit expression cycle is regulated by negative feedback which may be mediated both through TH activation by Dio2 as well as through TH destruction by Dio3. In the present study the rapid negative regulation of *Dio3* by TH administration supports thyroid hormone inhibition as a mechanism regulating *Dio3* expression and therefore controlling the cycles of pituitary TSH expression found in red drum. In the chicken pituitary the expression of *TSHR* has also been observed to increase as circulating THs increase and pituitary *TSH*β mRNA expression decreases [29], with the implication being that an intra-pituitary feedback loop is active in which TSH binding to the TSHR diminishes TSH expression. In my study the

expression of *TSHR* in the red drum pituitary was not regulated by TH immersion leaving the significance of TSHR in the red drum pituitary uncertain.

Treatment of fish with goitrogens (compounds that inhibit TH synthesis) consistently elevates TSH expression [55], as would be expected if negative feedback is active. Using the more sensitive qPCR technique, as compared to the dot blotting technique used by Cohn et al. [15], I was able to detect an elevation in the expression of the *GSU*α as well as *TSH*β subunits in MMI treated fish. Elevation in the expression of both TSH subunits under circumstances when both circulating hormones are depressed lends further support for negative feedback, although it does not help identify the differential effects from T_3 versus T_4. In this study, immersion with THs significantly inhibited pituitary *Dio2* expression while depression of circulating THs (MMI treatment) also paradoxically diminished *Dio2* expression. Given these results I conclude that *Dio2* mRNA expression in the red drum pituitary is variable using my experimental conditions, and while its presence must be required for its essential deiodinating role in T_4 feedback, the physiological regulation of the enzyme will need to be addressed by other molecular methods. In addition to changes in protein activity and mRNA expression contributing to alterations in deiodination activity, post-transcriptional regulation of deoidinases was previously postulated in the trout [6]. Differences in the relative importance of these multiple levels of regulation could contribute to the variable pituitary expression of *Dio2* in the red drum.

Blair et al. [4] hypothesized that the role of glycoprotein hormone receptors in non-endocrine tissues reflects an ancient paracrine function of these proteins. Co-expression of both TSH subunits and the TSHR in the gut of red drum could represent such an ancient function of TSH given that a similar expression pattern is seen in the mouse intestine where TSH paracrine signaling modulates immune function in response to infection [77, 88]. In the mouse intestine a local axis regulates immune cells by producing thyrotropin releasing hormone (TRH) which stimulates TSH synthesis within the epithelium, subsequently signaling proliferation of intraepithelial lymphocytes positive for the TSHR [40]. Mouse intestinal intraepithelial lymphocytes have been reported to show diminished proliferative response to TH treatment, possibly through a negative feedback effect on intestinal TRH and/or TSH expression [40]. Red drum intestinal *TSHβ* expression followed this same pattern of TRH stimulation by immersion and T_3 inhibition, by feeding, implying an intestinal axis similar to that in the mouse may regulate intestinal TSH in red drum. T_3 treatment may have been ineffective in the intestine in the immersion experiment because of the dosing protocol. Because TH tank water levels steadily decrease over time during static immersion, multiple treatments with T_3, similar to the TRH protocol, might have more effectively elicited the anticipated response. TRH treatment may also have been ineffective in the intestine in the feeding experiment for several reasons, including an inadequate dose, unknown time course of response, or destruction of the peptide hormone in the NaOH EtOH vehicle.

Because TSH signaling in the fish intestine might prove important for enhancing healthy fish populations, especially in captive and aquaculture applications, if the effect on immune function is conserved it thus deserves further investigation. Exogenous TH assimilation into the blood is regulated by peripheral deiodination in fish and mammals [19]. The up-regulation of intestinal *Dio3* in response to fed T_3 is consistent with a role for the intestine in protecting the fish from TH consumed as a component of prey.

In conclusion, I have shown that both THs can be absorbed from tank water by fish to yield physiological elevations of circulating hormones, eliciting rapid changes in thyroid-related gene expression. Most obvious among these genes are the two subunits for TSH, providing compelling evidence that both thyroid hormones can exert negative feedback at the pituitary, and supporting the hypothesis that the previously described T_4 cycle in red drum is at least partially driven by negative feedback. I have also shown that whereas THs had inconsistent effects on the expression of *Dio2*, the enzyme known to promote cellular actions of thyroid hormones in the pituitary gland, they do negatively regulate *Dio3* expression in the pituitary in a manner suggesting that negative thyroxine feedback on *Dio3* promotes the release of TSH subunits from TH inhibition and may be an important mechanism for generating daily thyroid hormone cycles. Together these results highlight the importance of central mechanisms at the pituitary in the regulation of fish thyroid function.

CHAPTER IV

CONCLUSIONS

Although peripheral regulation of thyroid function in fish is well established, its central regulation is still poorly understood. In mammals, T_4 secretion is principally driven by TSH from the pituitary gland [52]. Mammalian TSH is in turn regulated primarily by thyroid hormone negative feedback and hypothalamic stimulation [11] making TSH of primary importance in determining thyroid hormone availability to target cells in this central model [21]. In red drum, and possibly fish in general, circulating T_3 from peripheral production is regulated within a tight set point in the circulation, whereas T_4, the final output of central activation of the thyroid gland, fluctuates with a dynamic daily cycle [47]. The T_4 cycle in red drum is hypothesized to be initiated through a light and possibly food entrained central oscillator with an approximate 24h period [49], suggesting an important role for central mechanisms in the integration of sensory information to drive daily prohormone cycles. Conversely, the red drum daily T_4 cycle can be dampened by T_3 administration [49], supporting the hypothesis that TSH production in red drum is regulated by negative feedback at the pituitary gland. This hypothesis was the primary focus of my research. Because most TSH studies in fish have focused on hormone structure, this represents one of the few investigations of the physiological regulation of TSH in a teleost fish.

My research objectives as described in Chapter 1 were to test the hypothesis that in red drum daily thyroid hormone cycles are regulated centrally by TSH driving T_4 production. To address this hypothesis I first established qPCR techniques to quantify expression of TSH subunits. I additionally cloned and characterized fragments of the red drum deiodinase 2 and deiodinase 3. After developing novel qPCR assays to measure the mRNA expression of red drum *Dio2* and *Dio3*, I examined the relationship among expression of *TSH*β*, GSU*α*, Dio2,* and *Dio3,* and circulating T_4, concluding that the T_4 cycle in red drum is produced in part through feedback inhibition of *Dio3*-mediated TH inactivation, promoting T_3 availability for inhibition of TSH subunit expression. I next developed a TH immersion technique to study the regulation of *TSH*β*, GSU*α*,* and *Dio3* expression. These experiments demonstrated that physiological doses of TH can indeed rapidly down-regulate *TSH*β*, GSU*α*,* and *Dio3* expression. These experiments also demonstrated that T_4 administration can drive T_3 production and therefore possibly TH utilization. Evidence from this dissertation thus supports T_4 negative feedback, modulated through cyclical changes in pituitary deiodinase activity, as a mechanism regulating TSH-driven TH daily cycles in red drum, supporting the importance of central mechanisms of the pituitary in driving TH production and thus thyroid function in fish.

My studies are the first to demonstrate that the thyroid related transcripts *Dio2, Dio3*, and most notably *TSH*β*/GSU*α, are robustly expressed and

physiologically regulated in fish pituitary glands. These studies have advanced our understanding of fish thyrotropin function both by providing new information that thyroid-related pituitary gene expression in fish exhibits daily cyclicity and by suggesting new mechanisms for thyroid hormone negative feedback at the fish pituitary. Given that deiodinase expression in the pituitary is essential for T_4 feedback inhibition of TSH [14], my development of novel qPCR assays to measure the expression of the type 2 and 3 deiodinases has provided the first opportunity to establish that *Dio2* and *Dio3* are simultaneously expressed in fish pituitary glands. I next observed that the TSH subunits *TSH*β and *GSU*α exhibit an mRNA daily cycle of expression that precedes the daily T_4 cycle for captive red drum. As circulating T_4 increased TSH subunit expression decreased, supporting the proposed negative feedback of T_4 as a mechanism driving the TSH expression cycle. Although the TH-activating *Dio2* was expressed in the pituitary as was expected for feedback, it was not dynamically cyclic. Instead, the expression of pituitary TH inactivating *Dio3* cycled with the same temporal characteristics of the TSH subunits, supporting previous suggestions from salmonid studies that TH deactivation within thyroid hormone target tissues may be an essential component of the regulation of thyroid function in fish [6, 53]. My finding that rapid, physiological alterations in circulating thyroid hormone levels can elicit rapid responses in pituitary thyroid-related transcript expression, in particular thyroid-hormone induced depression of pituitary *Dio3* and TSH subunit expression, indicates that deiodinase 3 plays a central role in negative feedback

of T_4, and suggests that the daily cycle of circulating T_4 in red drum is the product of a peripheral-pituitary interaction that establishes a self-sustaining endocrine oscillator.

Based on these results I propose the following model for the regulation of daily cycles of TSH production in red drum. In this study I found evidence that as T_4 rises during the photophase of the red drum daily cycle the activating deiodinase *Dio2* is readily expressed, thus supplying T_3 available for feedback within thyrotrophs. The expression of the inactivating deiodinase *Dio3* in thyrotrophs decreases during the photophase, likely through direct thyroid hormone inhibition, to prevent destruction of the TH feedback signal. Thus, as circulating T_4 rises, negative feedback of T_3 formed through pituitary deiodinase 2 results in a decreased expression of the *GSU*α and *TSH*β subunits. This negative feedback is enhanced by the inhibition of deiodinase 3 by T_4. I propose that the decrease in mRNA expression of TSH would be followed 6-12 hours later by decreased TSH secretion and subsequent decreasing T_4 release throughout the scotophase. In common carp, salmon gonadotropin releasing hormone injection increased pituitary LHβ mRNA expression and circulating LH within 6 hours, indicating that changes in glycoprotein hormone mRNA can be associated with rapid subsequent changes in circulating hormone [38]. If TSH responds similarly, then a 6 hour lag could be expected for TSH mRNA expression to elicit an increased in circulating TSH. Based on mammalian studies, daily cycles of circulating TSH should precede TH release from the

thyroid gland by 1.5 to 4 hours [10, 73]. The 6-12 hour lag between TSH mRNA expression and circulating T_4 in red drum is therefore likely to be within a physiologically reasonable time frame. The diminished expression of *Dio3,* *GSU*α*,* and *TSH*β align with or lag slightly behind increasing circulating T_4 levels exemplifying T_4's proposed role in feedback. The expression of the inactivating *Dio3* then increases due to diminished T_4 inhibition during the scotophase. The resulting increased production of reverse T_3 and T_2 at the expense of T_3 in thyrotrophs thus diminishes inhibition of TSH subunit expression via T_3 negative feedback. This diminished feedback as circulating T_4 falls results in increased expression of the *GSU*α and *TSH*β subunits during the scotophase. I propose that the increase in mRNA expression of TSH would be followed (once again 6-12 hours later as predicted by my data) by increasing TSH secretion, driving the subsequent rise in circulating T_4 seen throughout the next photophase.

If enzyme activity (in the case of the deiodinases) and protein secretion (in the case of TSH), does lag behind mRNA expression by 6-12 hours as predicted by my research, then during times of TSH activation Dio3 would be up-regulated to disable THs, thus removing the negative regulator of TSH. TSH activation would result in T_4 secretion, which subsequently inhibits Dio3 expression. As Dio3 expression decreases, negative feedback on TSH increases, diminishing TSH, and consequently T_4 secretion. During times of TSH inactivation, Dio3 is down-regulated thus preventing the destruction of the TH negative TSH regulator. Negative feedback thus works in a temporal

sequence to generate a daily rhythm of intra-pituitary Dio3 activity, circulating TSH, and subsequent T_4 in red drum (figure 4.1). Because thyroid hormone deactivating Dio3 functions 180^o out of phase with T_4 negative feedback, this creates a self-sustaining oscillator resulting in daily cycles of TSH secretion and subsequent cyclic circulating T_4 (figure 4.1). Intriguingly, this oscillator as proposed could function without stimulatory input from a hypothalamic thyrotropin-releasing factor, consistent with the observation that hypothalamic regulation of TSH secretion in teleost fish may be predominantly under inhibitory control, as contrasted with the stimulatory control observed in tetrapod vertebrates [55]. Activation of this inhibitory hypothalamic control could also provide a mechanism for dampening of the amplitude of the oscillator observed during food deprivation (49).

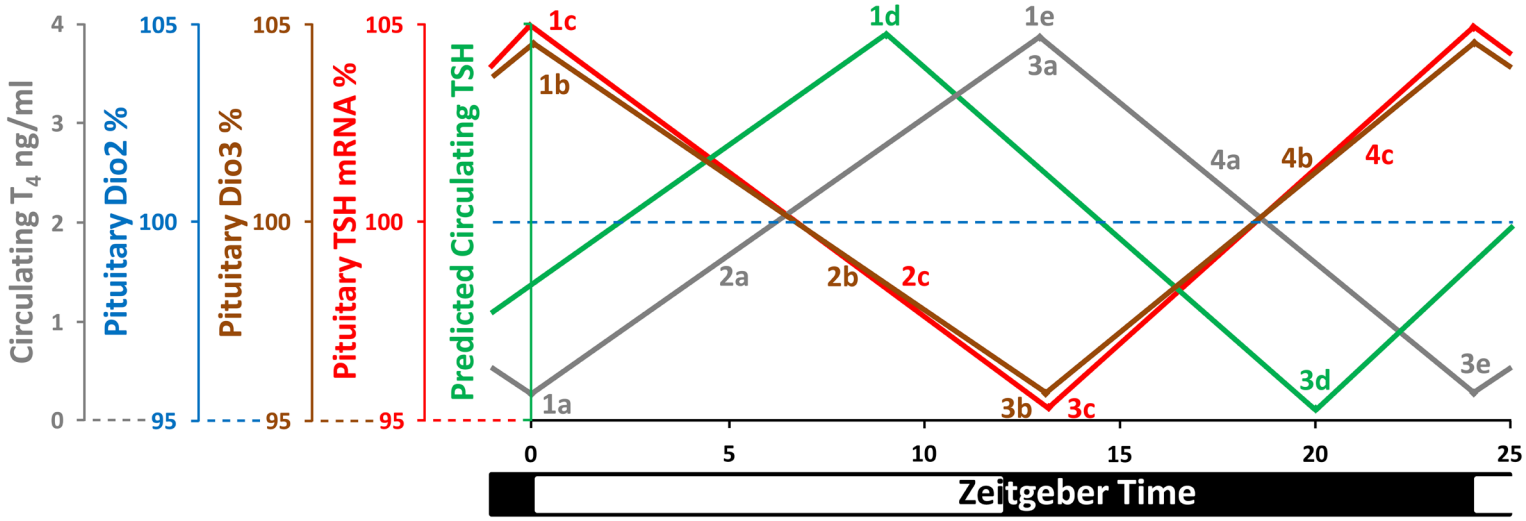

Figure 4.1. Proposed temporal relationship between daily cycles of circulating TSH, circulating T_4 and pituitary thyrotroph expression of TSH subunits and deiodinases in red drum. The activating deiodinase Dio2 is variable throughout both phases of the T_4 cycle but is nonetheless present as would be required for feedback regulation of TSH. 1. Maximal activation of TSH mRNA production. Early in the photophase low circulating T_4 (1a) coupled with maximal Dio3 activity (1b) drives intra-pituitary THs to their lowest levels. Minimal TH negative feedback allows for maximal TSH expression (1c). Circulating TSH (1d) is predicted to peak 6-12 hours later but 1.5-4 hours before highest stimulated T_4 release from the thyroid (1e). 2. Inhibition of TSH mRNA production. As time progresses in the photophase TSH mRNA expression is inhibited. Rising T_4 (2a) for negative feedback inhibits the expression of Dio3 (2b) allowing T_3 generated from T_4 through intra-pituitary Dio2 activity (as well as any T_3 which might enter thyrotrophs from the circulation) to access regulatory sites that negatively control TSH. As a result pituitary thyrotroph TSH production (2c) decreases. 3. Maximal inhibition of TSH mRNA production. Early in the scotophase T_4 reaches its zenith (3a) delivering maximum inhibition of Dio3 activity (3b) thus allowing intra-pituitary THs to reach their highest levels. Peaking TH negative feedback depresses TSH expression (3c) to its lowest with a predicted subsequent 6-12 hour later minimum in circulating TSH (3d) followed approximately 1.5-4 hours later by minimal T_4 release from the thyroid (3e). 4. Reactivation of TSH mRNA production. As time progresses in the scotophase expression of TSH mRNA is reactivated. Decreasing T_4 (4a) diminishes negative feedback, allowing the expression of Dio3 (4b) to increase, thus further decreasing intra-pituitary TH. Dio3 would thus prevent THs from accessing regulatory sites that negatively control TSH. As a result pituitary thyrotroph TSH production (4c) increases.

Although my study has provided a compelling argument for inhibition of pituitary TSH secretion by circulating thyroid hormones, confirmation of this model requires extension of these studies beyond gene expression to true measures of TSH secretion and deiodinase enzyme activity. An important extension of these studies would be to examine the relationship among mRNA expression of TSH subunits, TSH secretion, and subsequent T_4 release, which cannot presently be achieved due to the lack of techniques for measuring circulating TSH in fish. This linkage between TSH expression and protein secretion has received little attention in any species (although evidence does suggest that LH expression might be linked to secretion [38]), but is important to establish if one wishes to utilize subunit mRNA expression as an index of pituitary thyrotroph secretory activity. Similarly, my studies assumed that deiodinase expression reflects subsequent deiodinase activity in the pituitary, but this has rarely been examined in fish. However, in both tilapia [85] and trout [6], induced alterations in hepatic deiodinase expression were paralleled by changes in deiodinase enzyme activity, suggesting that much of the regulation of deiodinase function may be pretranslational in these species. Additionally, in rats, experimentally produced decreases in cerebral cortex *Dio2* mRNA expression were linked with decreased Dio2 enzyme activity lending further support that deiodinase mRNA expression is a useful index of deiodinase activity [8].

Leiner and MacKenzie [48] proposed that the red drum daily cycle of T_4 was circadian in nature and driven by a central oscillator requiring the hypothalamus to entrain the cycle to light, the pituitary to produce TSH, and the thyroid to produce T_4. T_4, as the ultimate output from this system, negatively regulates the axis to provide its nightly deactivation. My data support cyclic expression of pituitary thyroid-related transcripts regulated by rhythmic T_4 feedback, but does not address the underlying central circadian regulation via hypothalamic input which Leiner proposed to synchronize this cycle with photoperiod [46]. To address this question, future experiments, including *in vitro* studies, will be needed to establish the nature of hypothalamic regulation of TSH release from the pituitary. Studies in which communication between the hypothalamus and pituitary is disrupted could further demonstrate if hypothalamic input is needed to synchronize TSH release through stimulation (CRH or TRH) in the morning or inhibition (via a hypothalamic inhibiting factor?) in the evening. In spite of these uncertainties regarding hypothalamic control of TSH, my observations that *TSHβ* and *GSUα* mRNA are expressed in a manner reciprocal to the circulating T_4 cycle, that pituitary Dio3 may play an important role in the regulation of TSH expression by controlling intracellular availability of T_3 for negative feedback, and that THs can elicit rapid inhibition of pituitary TSH subunit expression as well as *Dio3* expression in the pituitary suggest that negative thyroxine feedback is an important mechanism for generating daily

thyroid hormone cycles, highlighting the importance of central mechanisms at the pituitary in the regulation of fish thyroid function.

REFERENCES

[1] Arjona FJ, de Vrieze E, Visser TJ, Flik G, Klaren PHM, Identification and functional characterization of zebrafish solute carrier Slc16a2 (Mct8) as a thyroid hormone membrane transporter, Endocrinology, 152 (2011) 5065-5073.

[2] Barca-Mayo O, Liao X-H, Alonso M, Di Cosmo C, Hernandez A, Refetoff S, Weiss RE, Thyroid hormone receptor α and regulation of type 3 deiodinase, Mol. Endocrinol., 25 (2011) 575-583.

[3] Bianco AC, Salvatore D, Gereben B, Berry MJ, Larsen PR, Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases, Endocr. Rev., 23 (2002) 38-89.

[4] Blair HC, Wells A, Isales CM, Pituitary glycoprotein hormone receptors in non-endocrine organs, Trends Endocrinol. Metab., 18 (2007) 227-233.

[5] Brabant G, Ocran K, Ranft U, Von Zur Mühlen A, Hesch RD, Physiological regulation of thyrotropin, Biochimie, 71 (1989) 293-301.

[6] Bres O, Plohman JC, Eales JG, A cDNA for a putative type III deiodinase in the trout (*Oncorhynchus mykiss*): Influence of holding conditions and thyroid hormone treatment on its hepatic expression, Gen. Comp. Endocrinol., 145 (2006) 92-100.

[7] Brokken LJS, Bakker O, Wiersinga WM, Prummel MF, Functional thyrotropin receptor expression in the pituitary folliculo-stellate cell line TtT/GF, Exp. Clin. Endocrinol. Diabetes, 113 (2005) 13,20.

[8] Burmeister LA, Pachucki J, St. Germain DL, Thyroid hormones inhibit type 2 iodothyronine deiodinase in the rat cerebral cortex by both Pre- and posttranslational mechanisms, Endocrinology, 138 (1997) 5231-5237.

[9] Bustin S, Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays, J. Mol. Endocrinol., 25 (2000) 169-193.

[10] Campos-Barros A, Musa A, Flechner A, Hessenius C, Gaio U, Meinhold H, Baumgartner A, Evidence for circadian variations of thyroid hormone concentrations and type II 5′-iodothyronine deiodinase activity in the rat central nervous system, J. Neurochem., 68 (1997) 795-803.

[11] Chiamolera MI, Wondisford FE, Thyrotropin-releasing hormone and the thyroid hormone feedback mechanism, Endocrinology, 150 (2009) 1091-1096.

[12] Chien J-T, Chowdhury I, Lin Y-S, Liao C-F, Shen S-T, Yu JY-L, Molecular cloning and sequence analysis of a cDNA encoding pituitary thyroid stimulating hormone β-subunit of the Chinese soft-shell turtle, *Pelodiscus*

sinensis, and regulation of its gene expression, Gen. Comp. Endocrinol., 146 (2006) 74-82.

[13] Chowdhury I, Chien JT, Chatterjee A, Yu JYL, *In vitro* effects of mammalian leptin, neuropeptide-Y, β-endorphin and galanin on transcript levels of thyrotropin β and common α subunit mRNAs in the pituitary of bighead carp (*Aristichthys nobilis*), Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol., 139 (2004) 87-98.

[14] Christoffolete MA, Ribeiro R, Singru P, Fekete C, da Silva WS, Gordon DF, Huang SA, Crescenzi A, Harney JW, Ridgway EC, Larsen PR, Lechan RM, Bianco AC, Atypical expression of type 2 iodothyronine deiodinase in thyrotrophs explains the thyroxine-mediated pituitary thyrotropin feedback mechanism, Endocrinology, 147 (2006) 1735-1743.

[15] Cohn W, Jones R, Valverde R, Leiner K, MacKenzie D, Molecular cloning and regulation of mRNA expression of the thyrotropin β and glycoprotein hormone α subunits in red drum, *Sciaenops ocellatus*, Fish Physiol. Biochem., 36 (2010) 1277-1290.

[16] Craig SR, MacKenzie DS, Jones G, Gatlin DM, Seasonal changes in the reproductive condition and body composition of free-ranging red drum, *Sciaenops ocellatus*, Aquaculture, 190 (2000) 89-102.

[17] Cyr DG, Eales JG, Interrelationships between thyroidal and reproductive endocrine systems in fish, Rev. Fish Biol. Fish., 6 (1996) 165-200.

[18] De Groef B, Van der Geyten S, Darras VM, Kühn ER, Role of corticotropin-releasing hormone as a thyrotropin-releasing factor in non-mammalian vertebrates, Gen. Comp. Endocrinol., 146 (2006) 62-68.

[19] Eales JG, Iodine metabolism and thyroid-related functions in organisms lacking thyroid follicles: Are thyroid hormones also vitamins?, Proc. Soc. Exp. Biol. Med., 214 (1997) 302-317.

[20] Eales JG, Modes of action and physiological effects of thyroid hormones in fish, In: Fish Endocrinology. Science Publishers, Enfield, NH (2006) 767-808.

[21] Eales JG, Brown SB, Measurement and regulation of thyroidal status in teleost fish, Rev. Fish Biol. Fish., 3 (1993) 299-347.

[22] Evans R, The steroid and thyroid hormone receptor superfamily, Science, 240 (1988) 889-895.

[23] Friesema ECH, Ganguly S, Abdalla A, Fox JEM, Halestrap AP, Visser TJ, Identification of monocarboxylate transporter 8 as a specific thyroid hormone transporter, J. Biol. Chem., 278 (2003) 40128-40135.

[24] Frith SD, Eales JG, Thyroid hormone deiodination pathways in brain and liver of rainbow trout, *Oncorhynchus mykiss*, Gen. Comp. Endocinol., 101 (1996) 323-332.

[25] Gan E-H, Quinton R, Physiological significance of the rhythmic secretion of hypothalamic and pituitary hormones, In: Prog. Brain Res., Elsevier, (2010) 111-126.

[26] Gensic M, Wissing PJ, Keefe TR, Mustafa A, Effects of iodized feed on stress modulation in steelhead trout, *Oncorhynchus mykiss* (Walbaum), Aquacult. Res., 35 (2004) 1117-1121.

[27] Goto-Kazeto R, Kazeto Y, Trant JM, Cloning and seasonal changes in ovarian expression of a TSH receptor in the channel catfish, *Ictalurus punctatus*, Fish Physiol. Biochem., 28 (2003) 339-340.

[28] Gregory CC, Porter TE, Cloning and sequence analysis of a cDNA for the β subunit of chicken thyroid-stimulating hormone, Gen. Comp. Endocrinol., 107 (1997) 182-190.

[29] Grommen SV, Geysens S, Darras VM, De Groef B, Chicken folliculo-stellate cells express thyrotropin receptor mRNA, Domest. Anim. Endocrinol., 37 (2009) 236-242.

[30] Han YS, Liao IC, Tzeng WN, Yu JY, Cloning of the cDNA for thyroid stimulating hormone β subunit and changes in activity of the pituitary-thyroid axis during silvering of the Japanese eel, *Anguilla japonica*, J. Mol. Endocrinol., 32 (2004) 179-194.

[31] Hanon EA, Lincoln GA, Fustin J-M, Dardente H, Masson-Pévet M, Morgan PJ, Hazlerigg DG, Ancestral TSH mechanism signals summer in a photoperiodic mammal, Curr. Biol., 18 (2008) 1147-1152.

[32] Hanon EA, Routledge K, Dardente H, Masson-Pévet M, Morgan PJ, Hazlerigg DG, Effect of photoperiod on the thyroid-stimulating hormone neuroendocrine system in the European hamster (*Cricetus cricetus*), J. Neuroendocrinol., 22 (2010) 51-55.

[33] Hayashizaki Y, Hiraoka, Y., Endo, Y., Miyai, K., Matsubara, K., Thyroid stimulating hormone (TSH) defiiciency caused by a single base substitution in the CAGYC region of the β-subunit., EMBO J., 8 (1989) 2291-2296.

[34] Higgs DA, Fagerlund UHM, McBride JR, Dye HM, Donaldson EM, Influence of combinations of bovine growth hormone, 17α-methyltestosterone, and L-thyroxine on growth of yearling coho salmon (*Oncorhynchus kisutch*) Can. J. Zool., 55 (1977) 1048-1056.

[35] Isorna E, Obregon MJ, Calvo RM, Vázquez R, Pendón C, Falcón J, Muñoz-Cueto JA, Iodothyronine deiodinases and thyroid hormone receptors regulation during flatfish (*Solea senegalensis*) metamorphosis, J. Exp. Zoolog. B. Mol. Dev. Evol., 312B (2009) 231-246.

[36] Ito M, Koide Y, Takamatsu N, Kawauchi H, Shiba T, cDNA cloning of the β subunit of teleost thyrotropin, Proc. Natl. Acad. Sci., 90 (1993) 6052-6055.

[37] Johnson KM, Lema SC, Tissue-specific thyroid hormone regulation of gene transcripts encoding iodothyronine deiodinases and thyroid hormone receptors in striped parrotfish (*Scarus iseri*), Gen. Comp. Endocrinol., 172 (2011) 505-517.

[38] Kandel-Kfir M, Gur G, Melamed P, Zilberstein Y, Cohen Y, Zmora N, Kobayashi M, Elizur A, Yaron Z, Gonadotropin response to GnRH during sexual ontogeny in the common carp, *Cyprinus carpio*, Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 132 (2002) 17-26.

[39] Kasper RS, Shved N, Takahashi A, Reinecke M, Eppler E, A systematic immunohistochemical survey of the distribution patterns of GH, prolactin, somatolactin, β–TSH, β–FSH, β–LH, ACTH, and α–MSH in the

adenohypophysis of *Oreochromis niloticus* , the Nile tilapia, Cell Tissue Res., 325 (2006) 303-313.

[40] Klein JR, Physiological relevance of thyroid stimulating hormone and thyroid stimulating hormone receptor in tissues other than the thyroid, Autoimmunity, 36 (2003) 417-421.

[41] Köhrle J, Local activation and inactivation of thyroid hormones: the deiodinase family, Mol. Cell. Endocrinol., 151 (1999) 103-119.

[42] Köhrle J, Thyroid hormone metabolism and action in the brain and pituitary, Acta Medica Austriaca, 27 (2000) 1-7.

[43] Larsen DA, Dickey JT, Dickhoff WW, Quantification of salmon α- and thyrotropin (TSH) β-subunit messenger RNA by an RNase protection assay: Regulation by thyroid hormones, Gen. Comp. Endocrinol., 107 (1997) 98-108.

[44] Larsen DA, Swanson P, Dickhoff WW, The pituitary–thyroid axis during the parr–smolt transformation of Coho salmon, *Oncorhynchus kisutch*: Quantification of TSHβ mRNA, TSH, and thyroid hormones, Gen. Comp. Endocrinol., 171 (2011) 367-372.

[45] Leary SC, Barton KN, Ballantyne JS, Direct effects of 3,5,3'-triiodothyronine and 3,5-diiodothyronine on mitochondrial metabolism in

the goldfish, *Carassius auratus*, Gen. Comp. Endocrinol., 104 (1996) 61-66.

[46] Leiner KA, Regulation of diurnal rhythms of circulating thyroid hormones in the red drum, *Sciaenops ocellatus*, Texas A&M University, (2000).

[47] Leiner KA, Han GS, MacKenzie DS, The effects of photoperiod and feeding on the diurnal rhythm of circulating thyroid hormones in the red drum, *Sciaenops ocellatus*, Gen. Comp. Endocrinol., 120 (2000) 88-98.

[48] Leiner KA, MacKenzie DS, The effects of photoperiod on growth rate and circulating thyroid hormone levels in the red drum, *Sciaenops ocellatus*: Evidence for a free-running circadian rhythm of T_4 secretion, Comp. Biochem. Physiol., A: Mol. Integr. Physiol., 130A (2001) 141-149.

[49] Leiner KA, MacKenzie DS, Central regulation of thyroidal status in a teleost fish: Nutrient stimulation of T_4 secretion and negative feedback of T_3, J. Exp. Zool., 298A (2003) 32-43.

[50] Lema SC, Dickey JT, Schultz IR, Swanson P, Thyroid hormone regulation of mRNAs encoding thyrotropin β-subunit, glycoprotein α-subunit, and thyroid hormone receptors α and β in brain, pituitary gland, liver, and gonads of an adult teleost, *Pimephales promelas*, J. Endocrinol., 202 (2009) 43-54.

[51] Lema SC, Nevitt GA, Evidence that thyroid hormone induces olfactory cellular proliferation in salmon during a sensitive period for imprinting, J. Exp. Biol., 207 (2004) 3317-3327.

[52] Li MD, Ford JJ, A comprehensive evolutionary analysis based on nucleotide and amino acid sequences of the α- and β-subunits of the glycoprotein hormone gene family, J. Endocrinol., 156 (1998) 529-542.

[53] Loter TC, MacKenzie DS, McLeese J, Eales JG, Seasonal changes in channel catfish thyroid hormones reflect increased magnitude of daily thyroid hormone cycles, Aquaculture, 262 (2007) 451-460.

[54] Luna M, Guzmn G, Navarro L, de la Peña SS, Valverde-R C, Circadian rhythm of type II 5'deiodinase activity in the rat hypothalamic-pituitary-adrenal axis, Endocrine, 3 (1995) 597-601.

[55] MacKenzie DS, Jones RA, Miller TC, Thyrotropin in teleost fish, Gen. Comp. Endocrinol., 161 (2009) 83-89.

[56] Manchado M, Infante C, Asensio E, Planas JV, Cañavate JP, Thyroid hormones down-regulate thyrotropin β subunit and thyroglobulin during metamorphosis in the flatfish Senegalese sole (*Solea senegalensis Kaup*), Gen. Comp. Endocrinol., 155 (2008) 447-455.

[57] Manzon RG, Denver RJ, Regulation of pituitary thyrotropin gene expression during *Xenopus* metamorphosis: Negative feedback is functional throughout metamorphosis, J. Endocrinol., 182 (2004) 273-285.

[58] Martin SAM, Wallner W, Youngson AF, Smith T, Differential expression of Atlantic salmon thyrotropin β subunit mRNA and its cDNA sequence, J. Fish Biol., 54 (1999) 757-766.

[59] McGoogan BB, Gatlin DM, Metabolic requirements of red drum, *Sciaenops ocellatus*, for protein and energy based on weight gain and body composition, The Journal of Nutrition, 128 (1998) 123-129.

[60] Miller TC, Biological activity of thyrotropin in two teleost fish, red drum (*Sciaenops ocellatus*) and goldfish (*Carassius auratus*), Texas A&M University, (2011).

[61] Moriyama S, Swanson P, Larsen DA, Miwa S, Kawauchi H, Dickhoff WW, Salmon thyroid-stimulating hormone: Isolation, characterization, and development of a radioimmunoassay, Gen. Comp. Endocrinol., 108 (1997) 457-471.

[62] Murakami M, There is a nyctohemeral rhythm of type II iodothyronine 5′-deiodinase activity in rat anterior pituitary, Endocrinology, 123 (1988) 1631.

[63] Nikrodhanond AA, Ortiga-Carvalho TM, Shibusawa N, Hashimoto K, Liao XH, Refetoff S, Yamada M, Mori M, Wondisford FE, Dominant role of thyrotropin-releasing hormone in the hypothalamic-pituitary-thyroid axis, J. Biol. Chem., 281 (2006) 5000-5007.

[64] Norris D, Vertebrate Endocrinology. 4th Edition ed., Elsevier Academic, San Diego, CA (2007).

[65] Orozco A, Valverde-R C, Thyroid hormone deiodination in fish, Thyroid, 15 (2005) 799-813.

[66] Pradet-Balade B, Burel C, Dufour S, Boujard T, Kaushik SJ, Quérat B, Boeuf G, Thyroid hormones down-regulate thyrotropin β mRNA level *in vivo* in the turbot (*Psetta maxima*), Fish Physiol. Biochem., 20 (1999) 193-199.

[67] Pradet-Balade B, Schmitz M, Salmon C, Dufour S, Quérat B, Down-regulation of TSH subunit mRNA levels by thyroid hormones in the European eel, Gen. Comp. Endocrinol., 108 (1997) 191-198.

[68] Prummel MF, Brokken LJS, Meduri G, Misrahi M, Bakker O, Wiersinga WM, Expression of the thyroid-stimulating hormone receptor in the folliculo-stellate cells of the human anterior pituitary, J. Clin. Endocrinol. Metab., 85 (2000) 4347-4353.

[69] Renshaw MA, Saillant E, Broughton RE, Gold JR, Application of hypervariable genetic markers to forensic identification of 'wild' from hatchery-raised red drum, *Sciaenops ocellatus*, Forensic Sci. Int., 156 (2006) 9-15.

[70] Ribeiro A, Ribeiro L, Sæle Ø, Dinis M, Moren M, Iodine and selenium supplementation increased survival and changed thyroid hormone status in Senegalese sole (*Solea senegalensis*) larvae reared in a recirculation system, Fish Physiol. Biochem., (Epub ahead of print, 2011) 1-10.

[71] Rocha A, Gómez A, Galay-Burgos M, Zanuy S, Sweeney GE, Carrillo M, Molecular characterization and seasonal changes in gonadal expression of a thyrotropin receptor in the European sea bass, Gen. Comp. Endocrinol., 152 (2007) 89-101.

[72] Rose MC, Voynow JA, Respiratory tract mucin genes and mucin glycoproteins in health and disease, Physiol. Rev., 86 (2006) 245-278.

[73] Russell W, Harrison RF, Smith N, Darzy K, Shalet S, Weetman AP, Ross RJ, Free triiodothyronine has a distinct circadian rhythm that is delayed but parallels thyrotropin levels, J. Clin. Endocrinol. Metab., 93 (2008) 2300-2306.

[74] Schmitz M, Balade B, Lebelle N, Dufour S, Querat B, *In vitro* study of the TSH subunit mRNA regulation in primary culture of pituitary cells of the

European eel, *Anguilla anguilla*, Ann. NY. Acad. Sci., 839 (1998) 466-468.

[75] Schreiber AM, Specker JL, Metamorphosis in the summer flounder (*Paralichthys dentatus*): Stage-specific developmental response to altered thyroid status, Gen. Comp. Endocrinol., 111 (1998) 156-166.

[76] Schweizer U, Weitzel JM, Schomburg L, Think globally: Act locally: New insights into the local regulation of thyroid hormone availability challenge long accepted dogmas, Mol. Cell. Endocrinol., 289 (2008) 1-9.

[77] Scofield VL, Montufar-Solis D, Cheng E, Estes MK, Klein JR, Intestinal TSH production is localized in crypt enterocytes and in villus 'hotblocks' and is coupled to IL-7 production: evidence for involvement of TSH during acute enteric virus infection, Immunol. Lett., 99 (2005) 36-44.

[78] Setlur S, Dighe R, Single chain human chorionic gonadotropin, hCGαβ: Effects of mutations in the α subunit on structure and bioactivity, Glycoconjugate J., 24 (2007) 97-106.

[79] Shupnik MA, Thyroid hormone suppression of pituitary hormone gene expression, Rev. Endocr. Metab. Disord., 1 (2000) 35-42.

[80] Shupnik MA, Ridgway EC, Chin WW, Molecular biology of thyrotropin, Endocr. Rev., 10 (1989) 459-475.

[81] Sohn YC, Yoshiura Y, Kobayashi M, Aida K, Seasonal changes in mRNA levels of gonadotropin and thyrotropin subunits in the goldfish, *Carassius auratus*, Gen. Comp. Endocrinol., 113 (1999) 436-444.

[82] Sower SA, Freamat M, Kavanaugh SI, The origins of the vertebrate hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) endocrine systems: New insights from lampreys, Gen. Comp. Endocrinol., 161 (2009) 20-29.

[83] Szkudlinski MW, Fremont V, Ronin C, Weintraub BD, Thyroid-stimulating hormone and thyroid-stimulating hormone receptor structure-function relationships, Physiol. Rev., 82 (2002) 473-502.

[84] Tata JR, Amphibian metamorphosis as a model for the developmental actions of thyroid hormone, Mol. Cell. Endocrinol., 246 (2006) 10-20.

[85] Van der Geyten S, Toguyeni A, Baroiller J-F, Fauconneau B, Fostier A, Sanders JP, Visser TJ, Kühn ER, Darras VM, Hypothyroidism induces type I iodothyronine deiodinase expression in tilapia liver, Gen. Comp. Endocrinol., 124 (2001) 333-342.

[86] VanPutte CLM, MacKenzie DS, Eales JG, Characterization of hepatic low-Km outer-ring deiodination in red drum (*Sciaenops ocellatus*), Comp. Biochem. Physiol. B: Biochem. Mol. Biol., 128B (2001) 413-423.

[87] Vischer HF, Bogerd J, Cloning and functional characterization of a testicular TSH receptor cDNA from the African catfish (*Clarias gariepinus*), J. Mol. Endocrinol., 30 (2003) 227-238.

[88] Wang J, Whetsell M, Klein JR, Local hormone networks and intestinal T cell homeostasis, Science, 275 (1997) 1937-1939.

[89] Wang Y, Zhou L, Yao B, Li C-J, Gui J-F, Differential expression of thyroid-stimulating hormone β subunit in gonads during sex reversal of orange-spotted and red-spotted groupers, Mol. Cell. Endocrinol., 220 (2004) 77-88.

[90] Watanabe T, Yamamura T, Watanabe M, Yasuo S, Nakao N, Dawson A, Ebihara S, Yoshimura T, Hypothalamic expression of thyroid hormone-activating and -inactivating enzyme genes in relation to photorefractoriness in birds and mammals, American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 292 (2007) R568-R572.

[91] Yamano K, Miwa S, Differential gene expression of thyroid hormone receptor α and β in fish development, Gen. Comp. Endocrinol., 109 (1998) 75-85.

[92] Yan HY, Thomas P, Histochemical and immunocytochemical identification of the pituitary cell types in three sciaenid fishes: Atlantic

croaker (*Micropogonias undulatus*), spotted seatrout (*Cynoscion nebulosus*), and red drum (*Sciaenops ocellatus*), Gen. Comp. Endocrinol., 84 (1991) 389-400.

[93] Yen PM, Physiological and molecular basis of thyroid hormone action, Physiol. Rev., 81 (2001) 1097-1142.

[94] Yoshiura Y, Sohn YC, Munakata A, Kobayashi M, Aida K, Molecular cloning of the cDNA encoding the β subunit of thyrotropin and regulation of its gene expression by thyroid hormones in the goldfish, *Carassius auratus*, Fish Physiol. Biochem., 21 (1999) 201-210.

APPENDIX

SUPPLEMENTAL FIGURES

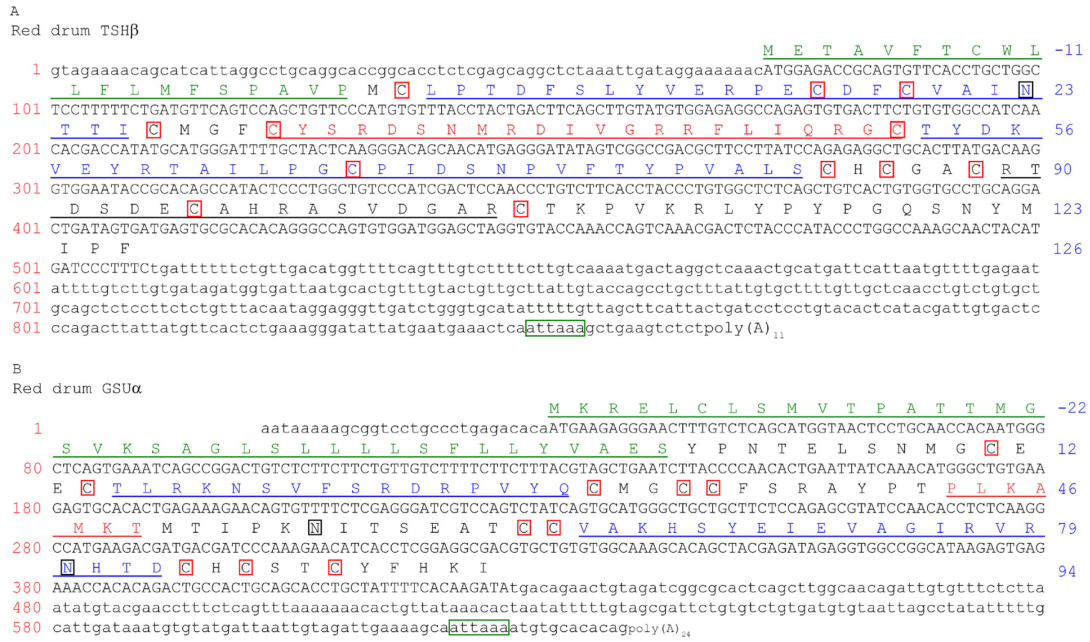

Figure A.1. Full length sequences of red drum (A) TSHβ and (B) GSUα. This figure is adapted from Cohn et al. [15]. See Chapter 2 for explanation of protocols used for the following descriptions. Using the unanalyzed sequence for the red drum TSHβ subunit from the authors of Cohn et al. [15], I cDNA cloned the red drum GSUα subunit. Internal cDNA fragments of red drum TSHβ and GSUα were generated using standard PCR with primers designed against conserved regions of the respective sequences. RACE PCR was used to generate full length sequences. These sequences were then aligned using Vector NTI (Invitrogen) software generating a consensus sequence for each gene product. Nucleotides shown in lowercase at the beginning and end of each sequence designate 5' and 3' UTR, respectively. Nucleotides designating coding sequence are indicated in uppercase. Nucleotides are specified by red numbers on the left. Single letter codes for each amino acid are designated above each corresponding codon and are specified by blue numbers at the right. All designated sites and sequences specified are presumptive. Functional regions of the cDNAs and proteins were predicted based on similar alignments by MacKenzie et al. [55]. Signal sequences are in green letters underlined in green, hair pin loops are shown in blue letters underlined in blue, and polyadenylation sites are boxed in green at the end of the 3' UTR. Invariant cysteines are boxed in red. A boxed "N" signifies a glycosylated asparagine as deduced from its alignment with glycosylated asparagines found in other TSHβ or GSUα proteins. For (A), the long loop is shown in red letters underlined in red and the seatbelt is emboldened and underlined in black. For (B) red letters underlined in red indicate an α-helix. Red drum TSH subunit sequences were most similar to sequences from other fish species.

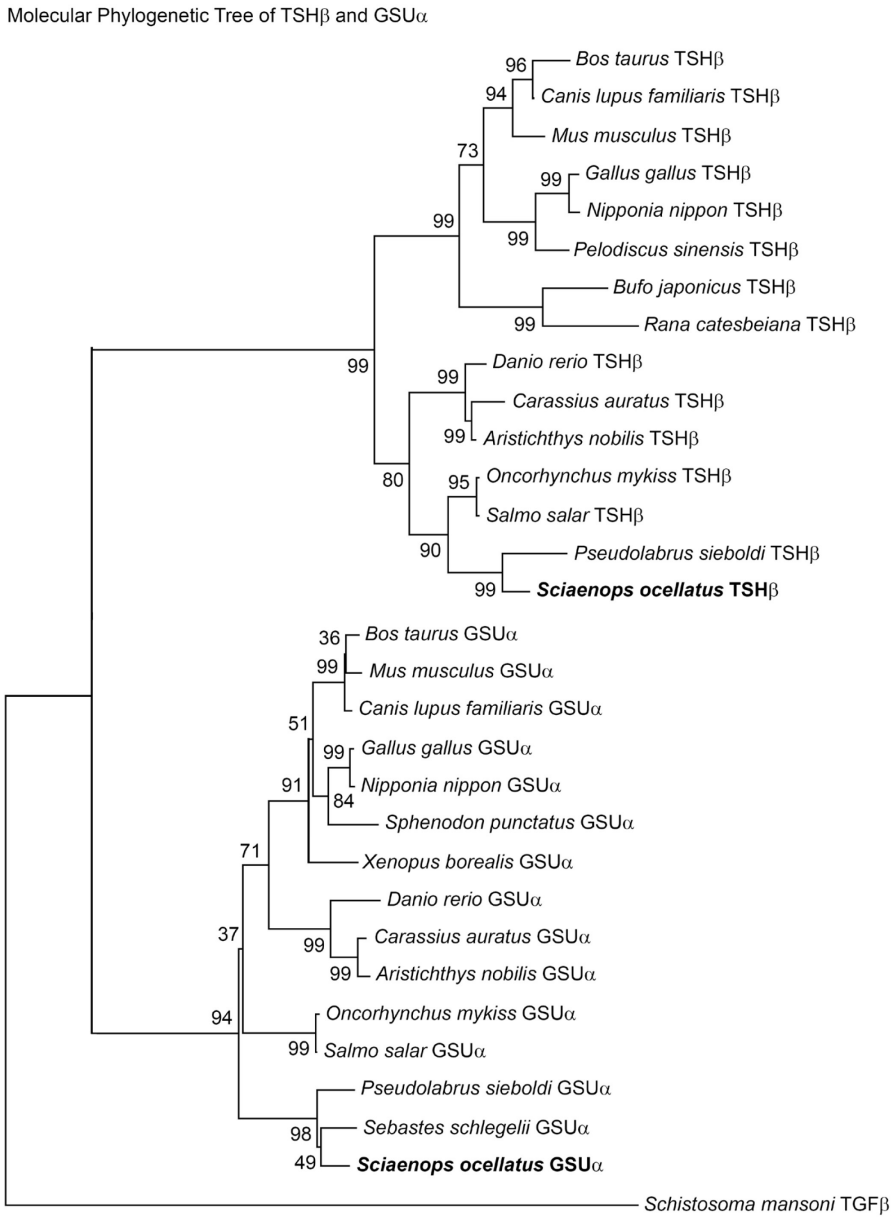

Figure A.2. Joint phylogenetic tree of red drum TSHβ and GSUα proteins. This figure is adapted from Cohn et al. [15]. See Chapter 2 for explanation of protocols used for the following data descriptions. A phylogenetic tree of selected vertebrate TSHβ and GSUα subunits was constructed by the Mega 4 software using the neighbor-joining method with 1000 replicates. *Schistosoma mansoni* transforming growth factor β (TGFβ) was chosen as a representative cystine-knot growth factor protein for the outgroup. The red drum sequences of interest are boxed within the phylogenetic tree. Numbers at branch points are bootstrap values. Red drum TSHβ segregated with other fish TSHβ proteins and branch paired with the other perciform species used in the analysis. Red drum GSUα segregated with other fish GSUα proteins and branch paired with other perciform species. These data once again signified that the red drum TSH subunits were most similar to sequences from other fish species.

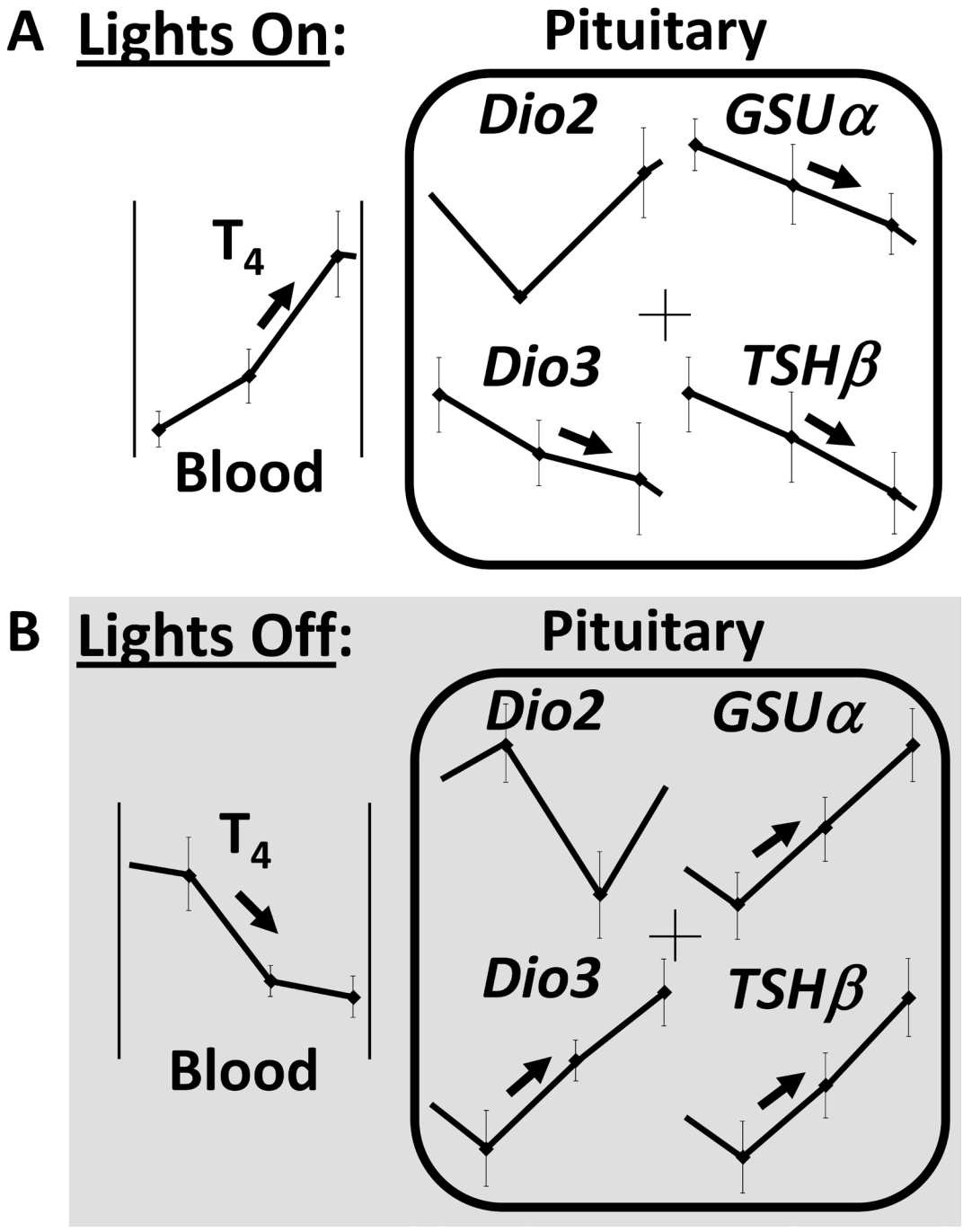

Figure A.3. Temporal changes in circulating T_4 and expression of pituitary thyroid-related genes. The activating deiodinase *Dio2* is highly variable throughout both phases of the T_4 cycle but is nonetheless present as required for feedback regulation of TSH. T_4 rises during the photophase (A) of the red drum daily cycle as the expression of both TSH subunits and the inactivating deiodinase *Dio3* in the pituitary decreases. During the scotophase (B), T_4 falls as the expression of the inactivating *Dio3* and both TSH subunits increases.

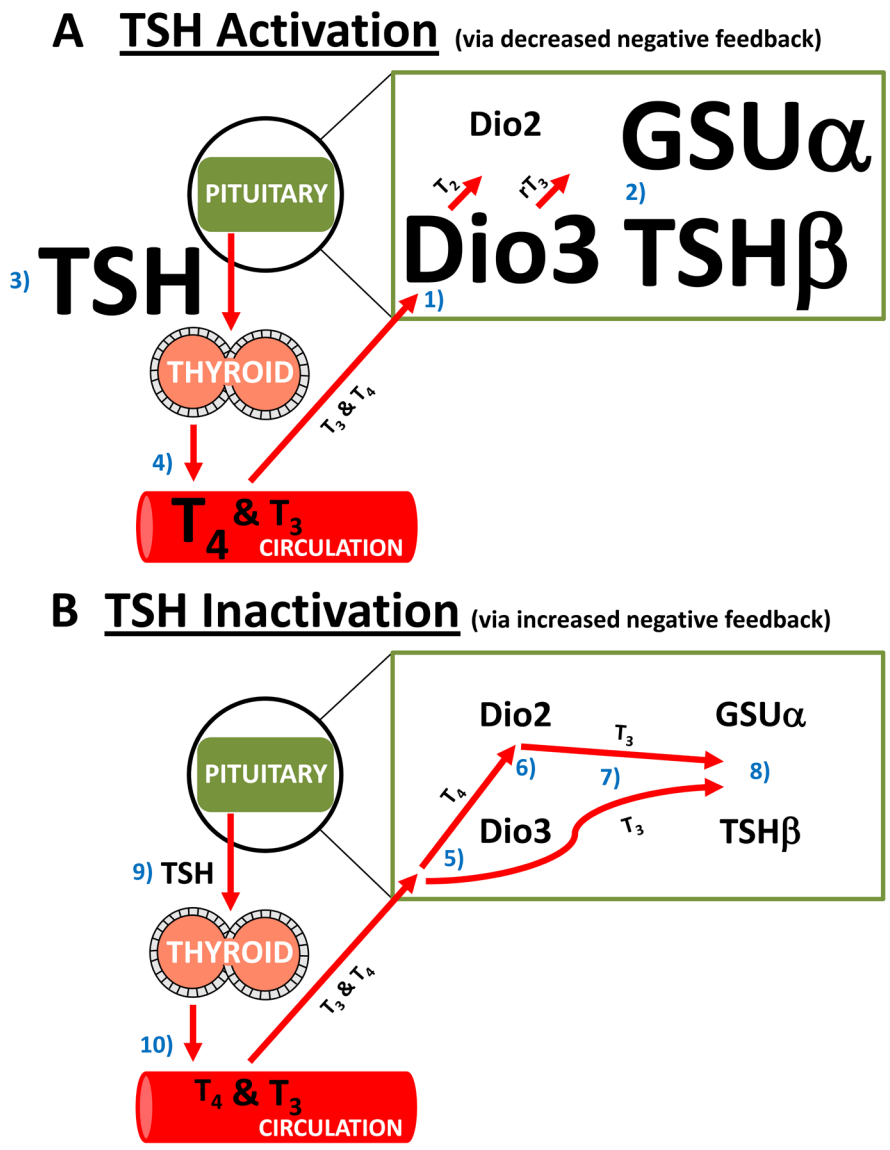

Figure A.4. Negative feedback regulation of TSH (A) activation and (B) inactivation in red drum. I propose that increasing TSH mRNA expression in the scotophase of the daily cycle would result in subsequent elevation 6-12h later of secreted TSH and TSH-dependent blood T_4. During TSH activation (A), 1) Dio3 would block THs, by deiodinating THs to inactive metabolites, from accessing regulatory sites that negatively control TSH. As a result thyrotroph 2) TSH production and 3) secretion increases, causing 4) increased T_4 release from the thyroid. To potentiate TSH activation, Dio3 activity in the pituitary prevents the thyroid released T_4 from negatively regulating TSH. Conversely, during TSH inhibition (B), 5) Dio3 activity is inhibited by circulating T_4, allowing 6) T_3 generated from T_4 through intra-pituitary Dio2 activity (as well as any 7) T_3 entering thyrotrophs from the circulation) to access regulatory sites that negatively control TSH. As a result thyrotroph 8) TSH production and 9) secretion decreases causing 10) T_4 release from the thyroid to diminish.

VITA

Name: Richard Alan Jones

Address: Department of Biology
College of Science
Texas A&M University
3258 TAMU
College Station, TX, 77843-3258

Email Address: rajones@mail.bio.tamu.edu

Education: A.S., Biology, Amarillo College, 1999
B.S., Zoology, Texas A&M University, 2001